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EVALUATION OF TERRESTRIAL MICROCOSMS FOR DETECTION, FATE, AND SURVIVAL ANALYSIS OF GENETICALLY ENGINEERED MICROORGANISMS AND THEIR RECOMBINANT GENETIC MATERIAL

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16. ABSTRACT

Terrestrial microcosms have been used for several decades by researcher to evaluate the fate and transport of xenobiotics in the environment; however, very little information is available on their application to the fate, survival, and transport of recombinant bacteria and microbial pest control agents. The research included in this document represents the current scientific information available regarding the applicability of terrestrial microcosms and related methodologies for evaluating detection methods and the fate and survival of microorganisms in the environment.

The three terrestrial microcosms described in this document were used to evaluate the survival and fate of recombinant bacteria in soils and in association with plant surfaces and insects and their transport through soil with percolating water and root systems, and to test new methods and procedures to improve detection and enumeration of bacteria in soil.

The usefulness of these various microcosms as a tool for risk assessment is underscored by the ease in obtaining soil from a proposed field release site to evaluate subsequent GEM fate and survival.

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ABSTRACT

Terrestrial microcosms have been used for several decades by researchers to evaluate the fate and transport of xenobiotics in the environment; however, very little information is available on their application to the fate, survival, and transport of recombinant bacteria and microbial pest control agents. The research included in this document represents the current scientific information available regarding the applicability of terrestrial microcosms and related methodologies for evaluating detection methods and the fate and survival of microorganisms in the environment.

The three terrestrial microcosms described in this document were used to evaluate the survival and fate of recombinant bacteria in soils and in association with plant surfaces and insects and their transport through soil with percolating water and root systems, and to test new methods and procedures to improve detection and enumeration of bacteria in soil. Simple (potting soil composed of peat mix and Perlite, lacking environmental control and monitoring) and complex microcosms (agricultural soil with partial control and monitoring of environmental conditions) were demonstrated to be useful tools for preliminary assessments of microbial viability in terrestrial ecosystems. These studies evaluated the survival patterns of *Enterobacter cloacae* (pBR322) in soil and on plant surfaces and the ingestion of this same microorganism by cutworms and survival in the foregut and frass. The Versacore microcosm design was used to monitor the fate and competitiveness of genetically engineered bacteria in soil. Both selective media and gene probes were used successfully to follow the fate of two recombinant *Pseudomonas* sp. introduced into Versacore microcosms. Intact soil-core microcosms were employed to evaluate the fate and transport of genetically altered *Azospirillum* sp. and *Pseudomonas* sp. in soil and the plant rhizosphere.

The usefulness of these various microcosms as a tool for risk assessment is underscored by the ease in obtaining soil from a proposed field release site to evaluate subsequent GEM fate and survival.

EXECUTIVE SUMMARY

The purpose of this document was to review and evaluate recent research regarding the applicability of terrestrial microcosms and related methodologies for evaluating detection methods, survival, and the fate of recombinant bacteria and microbial pest control agents in terrestrial environments. Although terrestrial microcosms have been used for several decades by researchers to evaluate the fate and transport of xenobiotics in the environment, very little information is available on their application to biotechnology risk assessment. There is currently a critical need to understand the advantages and limitations of microcosms for risk assessment and research applications. Microcosms can potentially provide a safe means for evaluating genetically engineered microorganisms (GEMs) before small-scale field testing.

The research selected for inclusion in this document represents the first scientific results that address this important aspect of risk assessment. The three terrestrial microcosms described in this document have been used to evaluate the survival and transport of GEMs in terrestrial systems and to test new methods and procedures for detection and enumeration of GEMs in soil. The utility of these microcosms is underscored by the ease in obtaining soil from a proposed field release site to evaluate GEM fate and survival. Because soil can contain local flora and insects and other invertebrates as well as natural microbial populations, it therefore conforms to the definition of microcosm or model ecosystem; the indigenous flora and fauna allow simulation of the natural environment for assessing survival of GEMs under contained, environmentally controllable situations.

Development and refinement of methods to monitor GEMs and their recombinant DNA in the environment are necessary because present techniques are not adequate for a critical analysis of microbial fate in the environment. Unlike chemicals, microorganisms must be differentiated from similar or identical species of indigenous microbes, and, therefore, it is desirable to be able to detect GEMs with techniques of the highest specificity and sensitivity. For these reasons, two recent advances that use DNA probes for detecting and enumerating GEMs in soils were selected for inclusion in this document.

The scientific literature contains a considerable amount of information on the use of terrestrial microcosms for evaluating chemical fate, transport, and effects that can provide a general understanding of the advantages and limitations of microcosms. Only limited aspects

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of this database, however, can be readily extended to GEM risk assessment because of fundamental differences between chemicals and living organisms. In Section 1.0, the key issues associated with GEM risk assessment are discussed, as are the advantages and limitations of terrestrial microcosms as they have been used for chemical risk assessment. However, the literature regarding the fate, transport, and effect of chemicals was not thoroughly reviewed in this document.

In Section 2.0, Assessing the Persistence of Recombinant Bacteria in Microcosms, it is shown that simple and complex terrestrial microcosms simulating agroecosystems are useful as tools for preliminary assessments of microbial viability in terrestrial ecosystems. The utility of microcosms for controlling environmental conditions and evaluating their effect on microbial survival on plant surfaces and in soil in microcosms is demonstrated with an *Enterobacter cloacae* strain containing the recombinant plasmid cloning vector pBR322. This research also presents some of the first information on the ingestion of GEMs by insects associated with plant surfaces and on the viability and subsequent dissemination of the microorganisms in the gut and frass.

In Section 3.0, The Fate of Recombinant Pseudomonads in Modified Soil-Core Microcosms (Versacores), use of a microcosm design termed the "Versacore" to monitor the fate and competitiveness of genetically engineered bacteria in soil is described. The major advantage to using repacked soil cores as microcosms is the relative ease of spatial and temporal sampling, which allowed an assessment of the movement of bacteria by water percolation and with plant roots.

In Section 4.1, Intact Soil-Core Microcosms for Evaluating the Fate and Survival of Genetically Engineered Microorganisms in the Environment, and Section 4.2, Fate of Root Growth-Inhibiting *Pseudomonas* sp. Tn5 Mutants in Intact Soil-Core Microcosms, intact soil cores were utilized as terrestrial microcosms for evaluating the fate and transport of genetically altered bacteria and the resultant ecologic effects. This microcosm design was used to assess the vertical transport of bacteria through soil, ingestion by earthworms and insects, survival in soil, and colonization of the rhizosphere. An advantage to this microcosm design is that soil cores are excised from the field, thereby maintaining ecosystem complexity and natural spatial distributions.

Sections 5.1 and 5.2 of this document represent the application of two recently developed methods employing DNA probes for monitoring GEMs in terrestrial environments. These two sections are similar in their use of DNA probes and the specificity of DNA:DNA hybridization for monitoring GEMs in soil, although they contrast in their approach. In Section 5.1, Detection of Two Engineered Pseudomonads and a Spontaneous Deletion Recombinant Derivative in Soil Using Gene Probes, the approach of direct extraction of DNA from soil was used to follow the fate of two engineered pseudomonads in soil. The direct extraction of DNA from soil has a major advantage in that it is independent of microbial culturability. This allows detection of the rDNA regardless of the source in the soil (i.e., in viable and nonviable host cells, recombined in the host organism, transferred to indigenous microorganisms, or existing free in protected sites in soil). The utility of this technique was demonstrated by the detection of a deletion recombination event in an engineered *Pseudomonas cepacia* strain released to the soil. In Section 5.2, Enumeration of Tn*5* Mutant bacteria in Soil by MPN-DNA Hybridization and Antibiotic Resistance, a most-probable-number approach accurately enumerated genetically altered bacteria in soil. This method proved to be sensitive and specific with a detection limit of approximately 10 cells/g soil for a *Pseudomonas putida* transposon Tn*5* mutant.

The advantages and limitations of the three microcosms evaluated in this document can be summarized as follows:

1. All the microcosms are useful for evaluating GEM survival in soil, rhizosphere, and rhizoplane; all can use soil taken from a proposed field site. At present, none of the microcosms has been calibrated to the field, although experiments are planned for 1989.

2. The Versacore is of limited size and would not be useful for evaluating the role of insects and earthworms in GEM transport and dispersal; because it is small scale, soil and plants may be significantly disturbed by regular sampling. However, replicate microcosms are easy to accommodate, and this is the least expensive test system.

3. The complex microcosm simulates tilled soil and can be used for aerosol, soil, or plant seed inoculation of GEMs; it can be used to study invertebrate roles in GEM dispersal; and strict environmental controls of soil moisture, relative humidity, and temperature are possible. Experiments that have been completed illustrate excellent microcosm-to-microcosm replicability.

4. The soil-core microcosm is especially useful for evaluating the horizontal transport and has the deepest columns of soil (60 cm), and soil leachates can easily be analyzed for GEMs as well as for chemical composition when evaluating GEM effects; also, insects and earthworms can be accommodated in the system.

On the basis of the limitations and advantages of the microcosm designs and research results presented in this document, some major conclusions have been drawn:

1. Terrestrial microcosms are an appropriate tool for biotechnology risk assessment of microorganisms, provided the operator understands the limitations of microcosms in general. (This is discussed further in Sections 1.0 and 6.0.)

2. The major advantages of the use of microcosms for biotechnology risk assessment are the ability to control and monitor environmental conditions and to contain the organisms to a higher degree than can be achieved in the field.

3. Field studies are necessary to calibrate microcosm behavior with the field to increase the utility of microcosms for predicting GEM behavior in release situations.

4. DNA probes can be highly specific and sensitive tools for monitoring the fate of GEMs and their recombinant DNA in terrestrial environments, but are presently limited by the intensive labor requirement relative to standard culturing techniques.

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1.0 INTRODUCTION

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ISSUES

Recent advances in molecular biology and genetic manipulation have allowed the development of microbial biotechnological products whose application requires release to the environment. Most of the products developed to date have been for agronomic applications and include ice nucleating-minus *Pseudomonas syringae*, *Rhizobium meliloti* with enhanced nitrogen-fixation capabilities, and *Pseudomonas fluorescens* containing *Bacillus thuringiensis* subsp. *kurstaki* delta-endotoxin (16). Other potential applications of genetically engineered microorganisms (GEMs) include biodegradation of hazardous organic waste, metal recovery from mining and waste effluents, and biological control of microbial, insect, and plant pests. The environmental applications of GEMs will certainly increase in the near future, and the potential risks associated with their environmental release have increased the need to understand more fully the fate and ecological effects of GEMs in the environment.

Microorganisms, once released, can be disseminated into the environment via different mechanisms that include aerial dispersal; surface and subsurface water transport; and plant, animal, insect, and avian vectors. In contrast to chemicals, microorganisms can increase in numbers under conditions that favor their growth. Although such conditions may be absent or diminished in the target environment, or the introduced strain may be unable to compete with the indigenous microflora, conditions conducive to survival or even growth may be present in environments to which microorganisms are transported. Genetic exchange between microorganisms, which also can occur by various mechanisms (13,14), has been documented in the environment; however, the environmental factors that promote genetic exchange and the consequences of such exchange are poorly delineated. Ecosystem effects are the most difficult risk assessment components to contend with but are among the most important.

The fate and transport of GEMs in the environment and their impact on the ecosystem are a function of their interaction with the physical, chemical, and biological components of that ecosystem. Therefore, assessment of the risks associated with environmental release of GEMs requires prior knowledge of their behavior in complex ecosystems, or some ability to predict such behavior. The ultimate level of ecologic complexity is the field, the real world itself, but conducting field experiments can involve numerous limitations.

Intermediate between "simple" laboratory bench media and field investigations are systems, termed model ecosystems or microcosms, that contain a few or many of the complexities of the natural environment yet are flexible to investigator control and manipulation of experimental variables. The term microcosm has been defined by Gillett and Witt (4) as ". . . a controlled, reproducible laboratory system which attempts to simulate the situation in a portion of the real world." Pritchard (11) offered a definition with several criteria to distinguish between microcosms and simpler laboratory studies:

> A microcosm is a calibrated laboratory simulation of a portion of a natural environment in which environmental components, in as undisturbed a condition as possible, are enclosed within definable physical and chemical boundaries and studied under a standard set of laboratory conditions. By calibrated, two things are meant: first, the simulation should be checked by comparing properties of the microcosm (functional processes, for example) with similar properties in the environment; second, the conditions in the microcosm or the results generated must be checked for their relation to the system design.

The key characteristic that allows microcosms to be considered as analogs of the environment is the interaction of biotic and abiotic components and processes. An advantage to using microcosms is that they contain fewer complexities, compared to the natural environment, that can prevent determination of the structure and functioning of an ecosystem. Simplifying natural environmental variability has disadvantages, however; ecosystem structure and function can be scale dependent, and process uncoupling can occur in systems that are greatly simplified.

Whether artificially constructed or excised from natural ecosystems, microcosms are experimental tools that are best defined by the questions or research hypotheses that are asked of them. To use microcosms for valid risk assessment, the researcher must be thoroughly familiar with their limitations and advantages.

BIOTECHNOLOGY RISK ASSESSMENT AND MICROCOSMS

The major components of risk assessment are defined by the events that could potentially follow the release of recombinant microorganisms to the environment. These events include the transport and dissemination of the released microorganisms, their survival, proliferation, and colonization in the target or nontarget environment (exposures), genetic recombination or transfer of genetic information to indigenous species, and, finally, deleterious effects on the indigenous biota and ecologic processes (effects). The U.S. Environmental Protection Agency (EPA) has focused considerable effort on developing methods that will assist in the safe release of GEMs (7). One of the major goals of the EPA biotechnology risk assessment program is to develop, refine, and evaluate methods for assessing dispersal and detection of GEMs (Figure 1). Microorganisms are not readily recalled once released, or easily disinfected in soils and sediments; thus, it is desirable to test, before large-scale field release, for fate, survival, gene transmission, and effects under conditions that provide a greater degree of containment than is possible in the field. Terrestrial microcosms as model ecosystems have the potential to provide test conditions that simulate certain aspects of the field. Further, microcosms provide the researcher with the ability to control and measure key environmental variables, such as moisture and temperature, that can have profound impacts on the survival and growth of microorganisms in the environment. This permits an evaluation of environmental conditions on GEM detection, fate, and transport that could not be controlled in field experiments. The Office of Technology Assessment (15) has outlined the stages of biotechnology risk assessment that include these components (Figure 2).

An important issue of biotechnology risk assessment is that of ecological effects. The questions that have been asked include "What constitutes an ecological effect?" and "What ecosystem properties should be protected?" The answers to these questions will, in part, dictate the ecosystem parameters to be evaluated before and after the release of GEMs to the environment. The specific concerns, which have been discussed elsewhere (1,3,15), include toxicity to nontarget species such as microbiota as well as higher animals, displacement of indigenous microbial species, leading to changes in community structure, and deleterious impacts on the cycling of nutrients and energy.

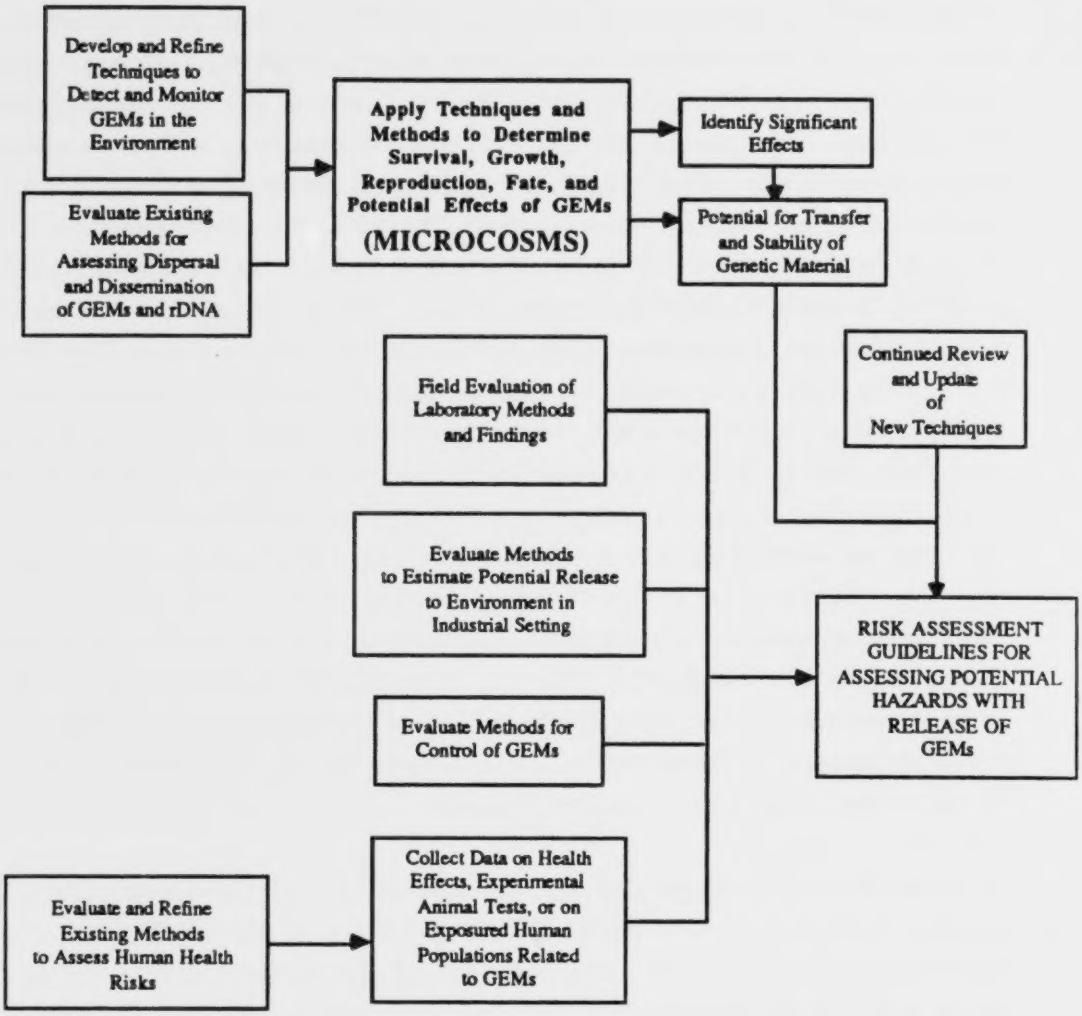
Develop and Refine Techniques to Detect and Monitor GEMs in the Environment
Evaluate Existing Methods for Assessing Dispersal and Dissemination of GEMs and rDNA
Apply Techniques and Methods to Determine Survival, Growth, Reproduction, Fate, and Potential Effects of GEMs
(MICROCOSMS)
Identify Significant Effects
Potential for Transfer and Stability of Genetic Material
Continued Review and Update of New Techniques
Field Evaluation of Laboratory Methods and Findings
Evaluate Methods to Estimate Potential Release to Environment in Industrial Setting
Evaluate Methods for Control of GEMs
RISK ASSESSMENT GUIDELINES FOR ASSESSING POTENTIAL HAZARDS WITH RELEASE OF GEMs
Evaluate and Refine Existing Methods to Assess Human Health Risks
Collect Data on Health Effects, Experimental Animal Tests, or on Exposured Human Populations Related to GEMs

Figure 1. EPA Biotechnology Risk Assessment Program. (Adapted from Levin et al. 1987. *Bio/Technology* 5:38-45.)

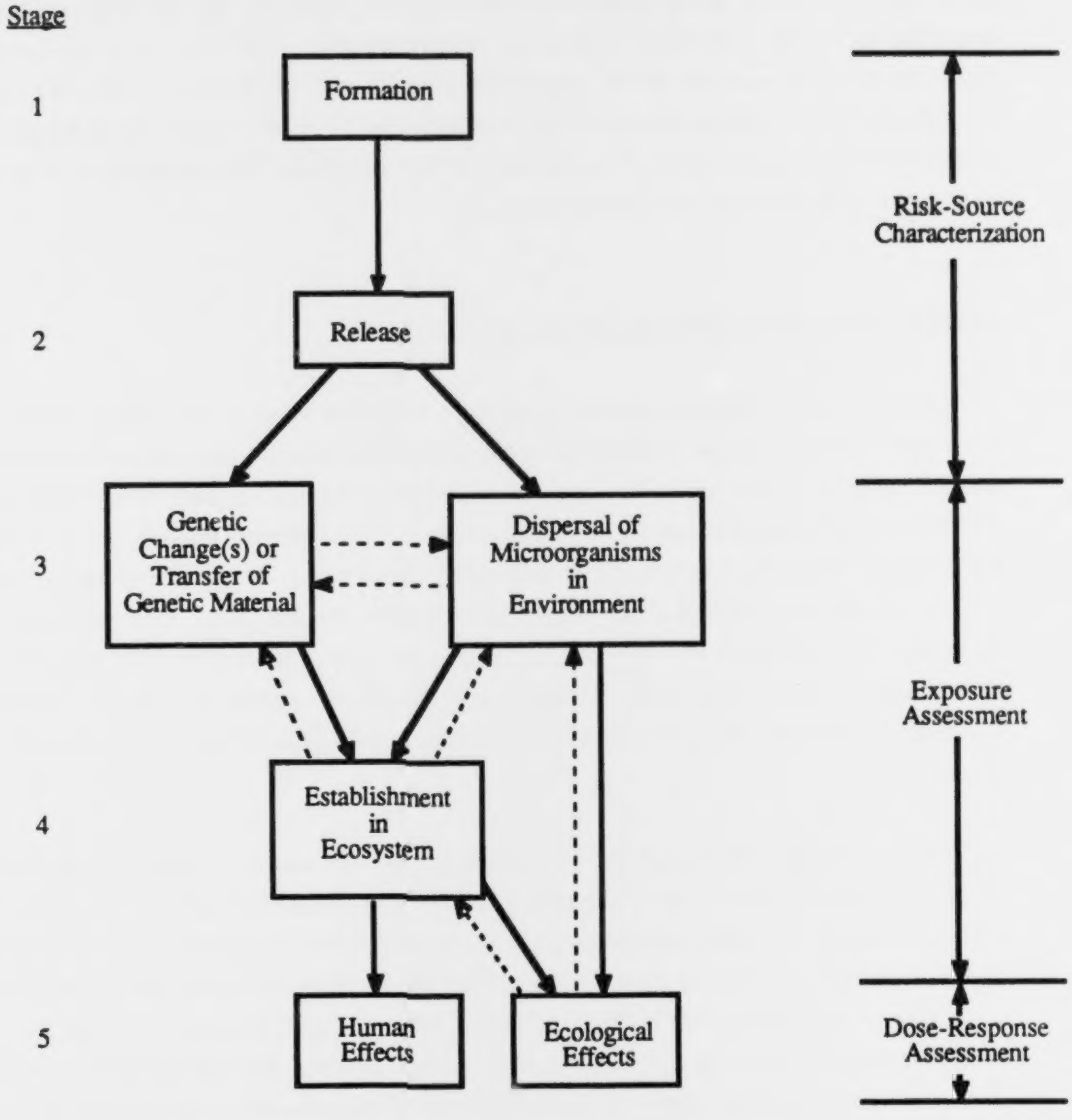
Stage
1
2
3
4
5
Formation
Release
Genetic Change(s) or Transfer of Genetic Material
Dispersal of Microorganisms in Environment
Establishment in Ecosystem
Human Effects
Ecological Effects
Risk-Source Characterization
Exposure Assessment
Dose-Response Assessment

Figure 2. Stages of biotechnology risk assessment. (From J. R. Fiksel and V. T. Covello. 1986. The suitability and applicability of risk assessment methods for environmental applications of biotechnology. In *Biotechnology Risk Assessment*, eds. J. Fiksel and V. T. Covello, pp. 1-34. Pergamon Press, New York.)

A necessary component of any microcosm or field evaluation of the ecological effects from the release of GEMs is the concurrent evaluation of fate, transport, and defining population densities so that dose-response curves can be constructed. Therefore, it is critical that sensitive, accurate methods be developed for tracking and enumerating GEMs and their recombinant DNA in the environment. DNA probes offer a detection specificity at the genetic level that was previously unattainable, and warrant investigation to increase their sensitivity and to automate processing of environmental samples.

ADVANTAGES AND LIMITATIONS OF MICROCOSMS

Relatively simple, defined systems containing soil, sediment, water, or artificial media are often used to obtain baseline information on the rates of biological transformation or ecological magnification of xenobiotics. Use of these materials for evaluating the fate and effects of GEMs in the environment can be criticized because of their limited relationship to natural ecosystems. This criticism is justified only when the objective or hypothesis being tested involves direct extrapolation of the results to the natural environment. Understanding the processes that influence the fate and transport of a chemical or microorganism in the environment requires, in general, experiments in simplified systems to avoid the complex interactions that make interpretation of experiments in more diverse systems difficult if not impossible.

Critics of using microcosms for biotechnology risk assessment of GEMs suggest that microcosms are elaborate, highly controlled systems that should fully predict or mimic the native ecosystem. In reality, microcosms can never fully mimic the natural environment, and critics argue that microcosms therefore are of little use in GEM risk assessment. Additional criticisms of microcosms have been variation between replicates and repeatability of experiments, disturbance as a result of sampling small systems, and spatial heterogeneities associated with using intact pieces of the environment as microcosms. Microcosms must have some ability to predict certain aspects of the field, even if they cannot fully mimic it, to be useful for risk assessment. Microcosms are a logical step in the study of systems of increasing complexity because they represent a higher order of complexity and therefore provide the researcher with a tool to evaluate GEM impacts on component interactions and ecological processes.

Microcosms or model ecosystems can have important applications in risk assessment for products of biotechnology, including use as field ecosystem analogs to address prerelease concerns regarding fate and effects. They may also have important uses as research tools to address specific questions requiring a system more complex than simple soil flasks or pots, tools controllable by the researcher yet not so complex that interpretation of results is difficult or impossible. Hill and Weigert (5) suggested two philosophies regarding the justification of microcosm research. The first is that microcosms are physical models or analogs that simulate all or part of an ecosystem; the second concept is that microcosms are actual ecosystems that include all or most of the properties of the field. The first philosophy is most appropriate for risk assessment, but both approaches are applicable.

Advantages of microcosms compared to field tests for evaluating the fate and impact from the release of GEMs include: the circumvention of large-scale environmental contamination; the use of the actual recombinant microorganisms, which cannot be recalled or be easily eliminated once released; and the ability to use radiolabeled chemicals. Additional advantages include ease of replication and use of appropriate controls; ability to include a variety of treatments and to perturb the system; ability to manipulate the trophic structure of the system; and control over system imports and exports (11).

The fate and transport of GEMs in the environment may not be accurately reflected by microcosm studies because of scale limitations and the inability to include biological, chemical, and physical heterogeneities that are characteristic of the natural system. The inability to predict the fate and transport of reactive solutes in subsurface environments is an example of how scale limitations can limit the applicability of microcosm results to the field. Transport models based on microcosm and process data are inadequate to rigorously predict the fate and transport of organic contaminants in the subsurface because of large vertical concentration gradients that can develop in aquifers (8). These gradients are likely the result of heterogeneities in the physical properties of the porous media (2) and in the distribution of microorganisms in the terrestrial subsurface (6,17).

Another scale-related limitation to microcosms is the exclusion of higher trophic levels, because these cannot be naturally maintained in microcosms. Inclusion of large plants or mammals in microcosms can result in extensive use of limited resources (e.g., nutrients, food sources) or physical destruction of the microcosm such that the balance of the system is dramatically upset and the microcosm thus no longer reflects the function and structures of its ecosystem analog. One such example, the introduction of meadow voles into terrestrial microcosms with subsequent devegetation (4), has been referred to as an "out-of-scale" process. Although devegetation by the voles did not directly affect the outcome of this pesticide fate experiment, it does illustrate the potential problems associated with introducing and attempting to maintain higher trophic levels in microcosms. Because of the limited volume of soil available for root exploration and the resulting imbalance in water and nutrient relationships in small pots, Ogunkunle and Beckett (9) found that yields of spring barley in undisturbed soil cores were more closely correlated with field trials than were pot studies. Although this problem is partially relieved by using larger terrestrial microcosms, increased rooting densities in the later stages of plant growth can result in rapid soil moisture loss, which must be compensated for to retain a robust plant community.

A further scale limitation imposed on microcosms is process uncoupling as a result of artificial boundaries and removal from the environment. Pritchard and Bourquin (12) suggested that ecosystems are biogeochemical units that integrate numerous metabolic networks and that microcosms reflect a proportion of these networks, but not all of them. Uncoupling does not necessarily eliminate critical processes or components but may influence the rates of processes. The impact, if any, of uncoupling processes on GEM risk assessment is unknown.

An important need in the application of microcosms for GEM risk assessment and regulation is the calibration or comparison of terrestrial microcosm behavior with the field. This lack of field calibration can lead to erroneous conceptions and conclusions. Field calibration is especially important if microcosms are to be used to predict the fate and effects of GEMs in the environment and that information is to be used for regulatory purposes. It is not necessary that microcosms behave in a manner identical to the field to be of use in risk assessment; however, it is important for processes and functions to show similar trends even if they differ significantly in magnitude. The answer to the question of how closely microcosms must mimic the field to be useful in risk assessment or research is currently unknown. Future research regarding the survival, transport, and ecological effects of GEMs in terrestrial microcosms and in the field will aid in answering this question.

Microcosms have been suggested as one tool in biotechnology risk assessment that can be used for investigating some of the fundamental questions associated with the release of GEMs to the environment (10). The inability to recall microorganisms or effectively mitigate their effects after they have been released to the environment requires a prerelease evaluation of GEMs that includes the various components of risk assessment. Because the history of microcosm development lies with chemical risk assessment, little information is available on microcosm use in biotechnology risk assessment. The unique properties of microorganisms necessitate the research and development of microcosms and microcosm protocols that are relevant to the environmental fate and effects of GEMs.

The purpose of this report is to summarize the current state of knowledge regarding the use of terrestrial microcosms for biotechnology risk assessment. Currently available information is limited and is scattered throughout the literature in peer-reviewed publications, and some is only given in sources with more limited distribution, such as reports or abstracts. The research presented in this report represents the first scientific information on the use of terrestrial microcosms for biotechnology risk assessment, compiled in a single citable document that permits assessing which microcosms are applicable for a particular purpose or release situation.

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2.0 ASSESSING THE PERSISTENCE OF RECOMBINANT BACTERIA IN MICROCOSMS

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INTRODUCTION

Interest in microcosm research has been renewed recently (5,12) by attention given to the release of genetically engineered microorganisms (GEMs) into the environment (8,10,14). As with toxic chemical testing, it is assumed that GEMs can be tested in microcosms before field release to determine a) survival in soil, on plants, and in association with animals; b) transport into higher trophic levels; and c) effects on the chemistry and biology of the targeted ecosystem. The general objectives of microcosm-based research with toxic chemicals are analogous to those of GEM research. Therefore, methods developed for chemical studies are pertinent to the newer applications of microcosms with GEMs. The review by Hammons (6) of the diversity of methods developed for studies of toxic chemicals is a thorough summary, including aquatic and terrestrial test systems, mathematical models, population interactions, and ecosystem effects.

The review by Gillett et al. (5) includes worthwhile information on the chief characteristics of several terrestrial microcosm types that have been applied to effects research with hazardous chemicals and discusses topics that are central to all microcosm experiments. They recommend using a) a mixed soil containing representative components from a field site; b) invertebrate species from at least three phyla; and c) at least one grassy and one broad-leafed plant. They also consider the use of a graded series in microcosm complexity, and express concern about the general lack of validation (precision) and verification (accuracy) of microcosm systems.

Parkes (11) emphasized the applications of microcosms to microbiological problems. Although the bacteria used in the reviewed studies were nonrecombinant strains, the basic issues are relevant to research with GEMs. In addition to the size and composition of the microcosm, Parkes discusses (a) the stabilization period that must precede collection of reliable data; (b) the source of the microbial inoculum and method of inoculation; and (c) the applicability of results to environmental questions. Van Voris (17) detailed a terrestrial soil-core microcosm that encloses a "piece" of intact soil containing indigenous assemblages of biota in a large polyethylene tube fitted with a Buchner funnel to allow leachate collection. The study of relatively undisturbed soil has advantages for testing effects on natural ecological processes that may be disrupted if the soil is mixed. Few available publications, however, specify microcosms as tools for the study of GEMs. Armstrong et al. (2) used a simple microcosm consisting of plants and cutworms in nonsterile potting soil to qualitatively assess bacterial survival in cutworms. The appearance of *Pseudomonas cepacia* (R388::Tn1721) was observed in the foreguts after larvae fed on sprayed leaves, but not in fecal pellets. The same type of microcosm was also used to verify a predictive model for the conjugative transfer of plasmids in the phyllosphere (7). As evidenced by the number of microcosm-related presentations at the REGEM Conference in Wales on the release of GEMs (13) and at the recent meeting of the American Society for Microbiology (1), many investigators are beginning to integrate microcosm methods into their experiments with recombinant microorganisms.

In line with the methods theme of this special report, the objective of this paper is to further discuss effective use of microcosms as tools for GEM studies. Therefore, an emphasis is placed on materials and methods, supplemented with several examples of applications. Included are descriptions of microbiological techniques for obtaining and processing microcosm samples from two microcosm designs that differ in methodological complexity.

MATERIALS AND METHODS

Design of Microcosms

Trays containing plants and soil (descriptions follow) were housed in a chamber (4) (Figure 1) consisting of a 1.0 x 0.75 x 0.75 m box with Pyrex glass top and plexiglass or glass walls, which rested on a polyethylene box measuring 1.0 x 0.75 x 0.55 m. Each chamber was irradiated with a 1000-W Sylvania metal halide lamp (positioned 55 cm above the box) on an 18:6 light:dark cycle. Air was forced through the chamber and exhausted through a HEPA filter (Astrocel, American Air Filter, Louisville, Kentucky). Room temperature was maintained at 22°C (±1°C) during the dark period and 23°C (±1°C) during the light period. A simple microcosm (lacking environmental monitoring and control; Figure 2) consisted of a plastic tray (27 x 55 x 5 cm) lined with a polyethylene bag containing potting mix [1 part peat potting mix to 1 part Perlite (Perlite Co., Portland, Oregon)] as described by Armstrong et al. (2).

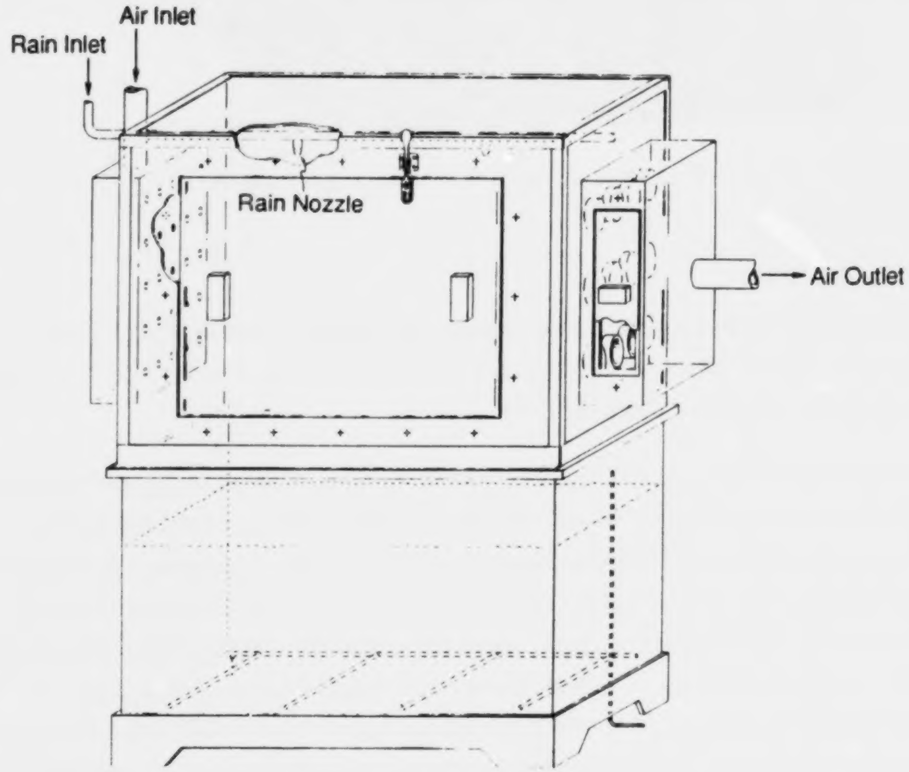

Figure 1. Schematic of microcosm chamber.

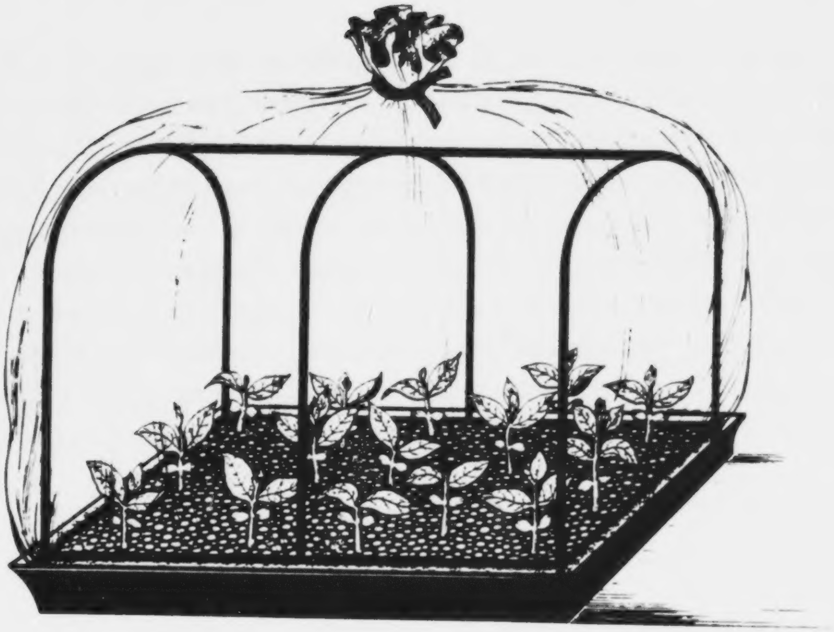

Figure 2. Schematic of simple microcosm.

In experiments with cutworm larvae, the bag was clamped near the top of a wire frame to stop escape of larvae. During experiments, trays were placed on a metal rack in the chamber described, supported 50 cm above the floor by bricks.

A second microcosm design was considered "complex" because the environment was monitored and partially controlled, and the soil was collected from a field site and not treated. This microcosm consisted of two wooden trays (47 x 37 x 7 cm) lined with polyethylene bags, each holding 6 to 7 cm soil (Hazelair-Veneta series; collected near Corvallis, Oregon). These trays were placed on metal racks in the chamber just described. Each chamber had a humidifier (#76850, Conviron Products of America, Pembina, North Dakota) below the rack. Water accumulating on the chamber floor during extended periods of humidification was suctioned through a tube, collected in a 5-gal container attached to a vacuum line, and disinfected with bleach.

Environmental Monitoring and Control

Environments of complex microcosms were monitored using a 21x micrologger (Campbell Scientific, Inc., Logan, Utah). Probes monitored air temperature and relative humidity (RH), soil temperature, soil moisture, and light. Probes were calibrated as follows. Each RH probe was wrapped in a moist paper towel and sealed in a plastic bag for 2 h to equilibrate to 100% RH. Correction factors were determined so that all probes would read 95 (equivalent to 100% RH according to the manufacturer) in the bags. Temperature probes were calibrated by immersion in a water bath. Correction factors were determined to within 0.1°C of a National Bureau of Standards calibrated thermometer. Soil moisture probes were placed in saturated soil (100% water-holding capacity, WHC), which was weighed as the soil dried (a moisture reading of -1 bar was equivalent to 62% WHC). Each chamber contained one temperature/RH probe (hanging 25 cm above the soil surface), two soil temperature probes (each buried 3 to 4 cm deep in the center of a tray), and two soil moisture probes (each near the soil surface in the center of a tray). Microloggers were programmed to collect environmental readings every minute and store hourly means. In studies of the effects of elevated RH, microloggers were programmed to turn humidifiers on and off to maintain RH near 90% during dark periods.

Bacteria and Culture Conditions

A strain of *Enterobacter cloacae* was isolated from the feces of a variegated cutworm (*Peridroma saucia* Hubner) collected in a peppermint field near Albany, Oregon. The isolate was identified with the API 20E identification system (Analytab Products, Plainview, New York). A spontaneous rifampicin- (Rif-) resistant mutant isolate was transformed (9) with plasmid pBR322, which encodes oxytetracycline (Otc) resistance. Bacteria were cultured in LB broth or agar-based medium (9) amended with three antibiotics (Rif, 100 µg/ml; Otc, 15 µg/ml; cycloheximide, 25 µg/ml; Sigma Chemical Co., St. Louis, Missouri), which effectively inhibited growth of all bacteria and fungi indigenous to leaves and soil. To enumerate colonies, samples were serially diluted in phosphate buffer (1.2 m*M* KH_2PO_4, pH 7.2), spread in duplicate on LB agar plus antibiotics, and incubated 20 h at 30°C. Colony counts from a pair of plates were averaged for calculations of colony-forming units (CFU).

Plant Growth Conditions

In the simple microcosm, 50 bean seeds (cultivar Blue Lake Bush) were planted in five rows per tray. For studies with complex microcosms, two seeds were planted in each of 12 holes per tray and one plant was thinned from each pair after 1 week. Plants were raised under 40-W fluorescent lamps (Sylvania Cool White, F40/CW) on a 18:6 light:dark cycle. They were watered/fertilized with 2 liters of nutrient solution (North Carolina State University phytotron nutrient solution lacking hampol copper and sequestrene cobalt) (3) per tray every 48 to 72 h. After the first appearance of flowers (about 4 weeks), plants were used in experiments. Three days before an experiment, trays were placed in a microcosm chamber to expose the plants to the experimental environment.

Insect Growth Conditions

Variegated cutworm larvae were collected from fields near Corvallis, Oregon, and raised from eggs in Petri dishes containing Diet 9000 (15) (Bioserve Inc., New Jersey), which had been sterilized by autoclaving for 15 min. To coordinate maturation of larvae and plants for experimental use, larval growth was slowed or quickened by incubation at temperatures between 12° and 30°C. Fifth- and sixth-instar larvae were transferred to fresh medium every 48 h. Late sixth-instar larvae were then transferred to medium 9000 in 28-g (1-oz) plastic containers, where they pupated. Pupae were put in Petri dishes inside a wire mesh cage (enclosed in a plastic bag with a damp sponge to increase RH) and incubated at 25°C with an 18:6 light:dark cycle. Emergent moths deposited eggs on paper towels in the cage. Egg masses were transferred to Petri dishes and incubated at 25° to 30°C for 4 to 6 days. Within 18 h of emergence, first-instar larvae were placed on Diet 9000. Bactericidal chemicals in the diet were removed from the insects by feeding them fresh plants for 3 days before microcosm studies.

Introduction of Organisms into Microcosms

A spray inoculum was prepared from bacteria cultured for 15 to 18 h at 30°C with shaking in LB broth containing Otc and Rif. Cells were pelleted by centrifugation (10 min at 3500 x *g* at 4°C), suspended in 1.2 m*M* phosphate buffer, centrifuged again, and diluted 100 fold in phosphate buffer to about 2 to 5 x 10^7 CFU/ml. Microcosm contents were sprayed within the first 4 h of a light period. To contain escape of aerosolized bacteria, sprays were done inside a negative air flow hood with a HEPA filter on the exhaust port. Plants were sprayed to runoff (about 100 ml of suspension per tray) using a plastic misting bottle (rinsed with 95% ethanol followed by sterile phosphate buffer). In studies with insects, about 40 larvae were introduced 2 h after the spray. Following experiments, soil and plants were autoclaved and the inner surfaces of the microcosm boxes disinfected with dilute bleach.

Bacterial Transport Studies

For 24 h, cutworms were fed bean plants sprayed with bacteria and then transferred to uninoculated plants. Foregut, leaf, and soil samples were taken daily for 3 days.

Sample Collection and Processing

In studies of ingestion of bacteria by larvae, three samples of plants, soil, foreguts, and frass were collected on each sampling day. Plant samples (weighing about 1.5 to 3.5 g) consisted of one cotyledon, one first trifoliate, and one second trifoliate. These were aseptically collected at random, put in sterile plastic bags holding 20 ml phosphate buffer, and blended for 1 min in a Stomacher blender (Model 80, Tekmar Co., Cincinnati, Ohio). Using a spatula, soil samples of about 0.4 to 1 g each were aseptically collected at random from the top centimeter of soil. These were vortex mixed for 1 min in 18 x 150 mm screw-capped tubes containing 5 ml phosphate buffer and seven glass beads (5 mm in diameter). To sample a larval foregut, the head was removed with scissors and the foregut (weighing about 0.1 to 0.4 g) pulled out with forceps (2). The sample was vortex mixed for 1 min in an 18 x 150 mm screw-capped tube containing glass beads and 5 ml of phosphate buffer. A frass sample was aseptically collected with forceps as a pellet was extruded by an insect (2). Each pellet (about 5-50 mg) was placed on Parafilm, weighed, and dispersed for 30 sec with a Pellet Pestle (Kontes, Vineland, New Jersey) in a 1.5-ml Eppendorf tube containing 0.05 ml phosphate buffer, which was followed by addition of 0.95 ml of phosphate buffer and vortex mixing for another 30 sec.

In bacterial transport studies, three plant, three soil, and six foregut samples were taken each day after transfer of insects. Samples were processed as described except 10 m*M* Tris buffer, pH 7.5, was used instead of phosphate buffer and leaves were blended in 12.5 ml buffer. From each complex microcosm, six plant samples (three per tray) and six soil samples (three per tray) were taken on a sampling day. On the day of the spray, samples were collected 4 to 6 h after the spray, when the leaf surfaces were dry. Samples were processed as described for studies of ingestion of bacteria.

Data Analysis

Bacterial survival was plotted as the mean of the logarithm (log) CFU/g fresh weight of sample. Bars around each mean represent the standard error of the mean (Figure 3). In statistical comparisons of log-transformed data from pairs of complex microcosms, analyses were performed using a two-way factorial, completely randomized block design with microcosm and day as the two factors (16). Slopes (log CFU/g per day) for complex microcosm data from days 5 through 21 were computed by regressing a least-squares analysis of log CFU/g versus day. Calculations were done with the SAS General Linear Methods procedure.

RESULTS AND DISCUSSION

Use of the Simple Microcosm to Study Ingestion of Bacteria by Insects

The simple microcosm was useful in exploratory development of methods, despite the possible disadvantages of a shallow soil bed, the type of soil used, and the absence of sensors to record environmental parameters. This microcosm design was effective in studies of ingestion of recombinant bacteria by insects. *Enterobacter cloacae* (pBR322) was sprayed on soil and plants and then tracked in the digestive systems of cutworms feeding on these plants. The bacterial populations associated with soil and leaves persisted at 10^5 to 10^6 CFU/g for as long as 14 days (Figure 3), and in the digestive tracts at nearly 10^5 CFU/g and in the frass at about 10^6 CFU/g (Figure 4). These large populations of *E. cloacae* (pBR322) in foreguts and frass indicated that this species survived well during passage through the gut.

Simple microcosms were also used to study site-to-site transport of bacteria associated with insects that were fed foliage sprayed with *E. cloacae* (pBR322) and then transferred to plants that had not been inoculated. On the day of transfer of larvae, foreguts contained approximately 10^6 CFU/g (Figure 5). These populations declined slightly by day 3, and the numbers on leaves and soil increased to about 10^5 and 10^6 CFU/g, respectively. Because the larvae were not surface sterilized before the transfer, it is not known what proportion of the *E. cloacae* (pBR322) may have been transported via the surfaces of insects. However, regardless of the location of the bacteria, the results illustrate the active role of cutworms as carriers of these bacteria.

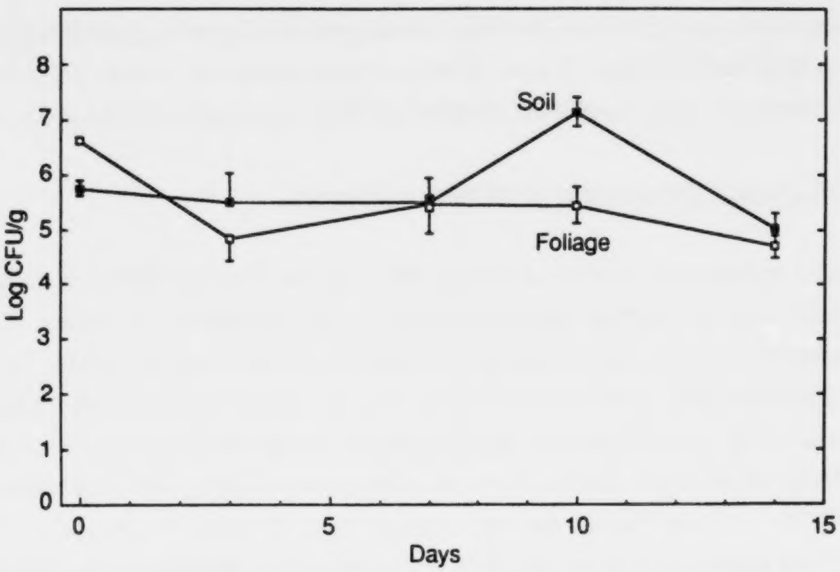

Figure 3. Persistence of *Enterobacter cloacae* (pBR322) sprayed on bean plants and soil in simple microcosm containing cutworms.

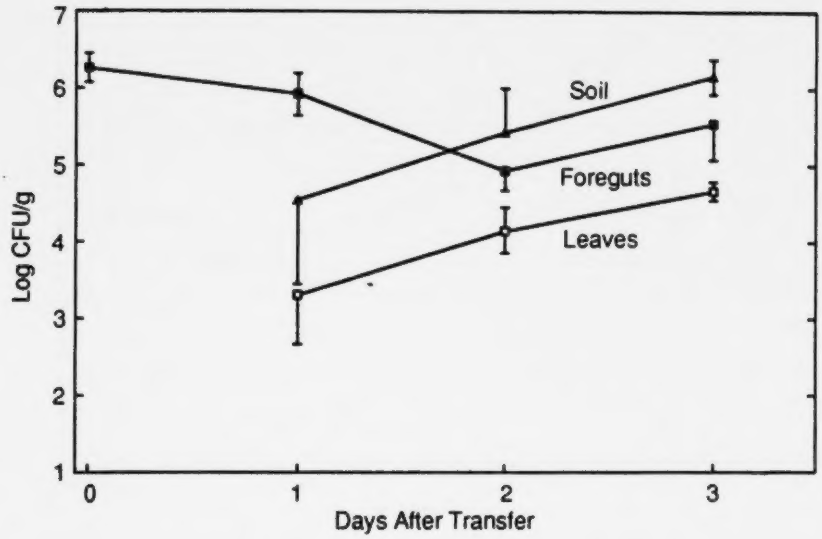

Figure 4. Appearance of *Enterobacter cloacae* (pBR322) in foreguts and frass of cutworms transferred to simple microcosm containing bean plants and soil sprayed with *E. cloacae* (pBR322).

Other possible applications of the simple microcosm include investigations of a) survival of bacteria in other invertebrates, b) survival of recombinant bacteria in insects during pupation and adult emergence, and c) regrowth of bacteria in frass deposited on leaves and in soil.

Use of the Complex Microcosm to Study Bacterial Survival

Complex microcosms contained natural soil from the field and included environmental probes, permitting monitoring and partial control of environmental conditions. This design was developed to assess the impact of environment on survival of GEMs. Its utility is illustrated by three trials in which the environments of a pair of chambers were maintained as closely as possible. These "baseline" experiments were necessary to establish information on the similarity of survival curves between microcosms during each experiment and the reproducibility of these curves between experiments. In trials 1 through 3, *E. cloacae* (pBR322) was sprayed on plants and soil in two chambers and the populations monitored.

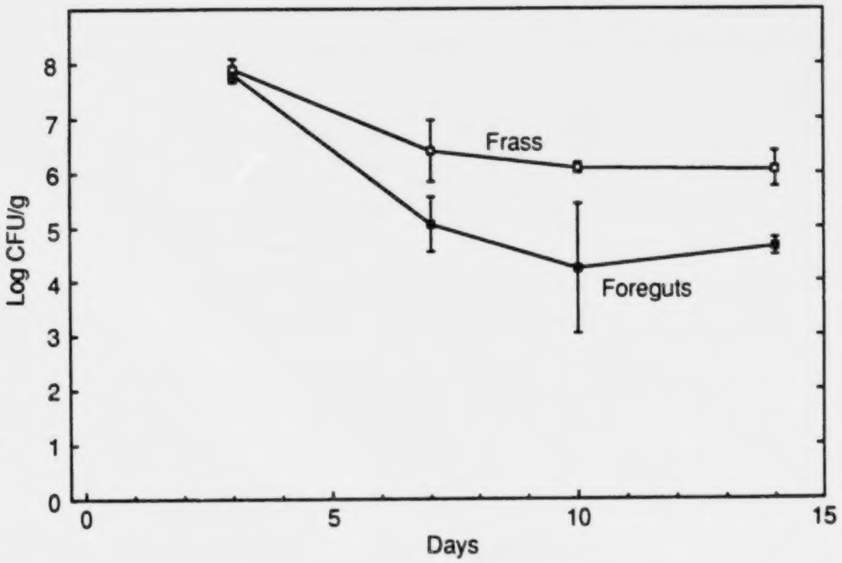

Figure 5. Appearance of *Enterobacter cloacae* (pBR322) in an uninoculated simple microcosm after the introduction of cutworms that were fed bean leaves sprayed with these bacteria.

Figure 6 depicts the environmental data collected from two such microcosms in trial 1. Figure 6A displays the changes in RH, which were achieved by programming the micrologger to turn humidifiers on during the dark. The RH dropped to 45% to 55% during the day when humidifiers were off. The mean hourly RH values in microcosms 1 and 2, respectively, were as follows: dark period, 90% [standard deviation (SD), 6%)] and 88% (SD, 8%); light period, 57% (SD, 7%) and 56% (SD, 7%). The soil moisture (data not shown) was between 62% and 100% WHC for 73% of the time in microcosm 1 and 60% of the time in microcosm 2.

Figure 6B illustrates the typical cyclic pattern of chamber air temperature, fluctuating between 19° and 34°C during the daily cycle with respective day and night mean hourly values of 30°C (SD, 1°C) and 22°C (SD, 1°C) in both microcosms. These changes were caused by heat from the lamps. Figure 6C shows the soil temperatures recorded by thermistors in the four trays. These data are presented to illustrate the type and extent of daily variations. Because soil temperature readings depended on thermistor depth in the soil and the density of the foliar layer, the readings varied considerably among probes. During the dark period, soil temperature typically approached the air temperature; during the light period, the soil was usually about 5°C cooler than the air. Table 1 summarizes the RH, soil moisture, and air temperature data for all experiments reported here. The relative consistency of environmental data from trials 1 through 3 indicates the repeatability of chamber environments and their utility for comparative tests.

Figure 7 presents the number of *E. cloacae* (pBR322) recovered from plants in both microcosms during trial 1. The curves depict the biphasic die-off that was typical for epiphytic populations of *E. cloacae* (pBR322). During the 5 days after the spray, the detectable numbers decreased more rapidly than during days 5 through 21. Only 1% of the initial population was recovered 5 days after a spray. As indicated in Table 2, the slopes of the curves during days 0 through 5 were -0.33 in microcosm 1 and -0.32 in microcosm 2, respectively. These contrasted with the slower die-off between day 5 and day 21 (slopes: microcosm 1, -0.03; microcosm 2, -0.06). Figure 8 shows the changes in *E. cloacae* (pBR322) populations in the soil during trial 1. About 10% of the initial soil population was detected on day 5 (slopes for days 0 through 5: microcosm 1, -0.16; microcosm 2, -0.21; Table 2). By day 21, only 10% of the day 5 population was detected (slopes for days 5 through 21: microcosm 1, -0.06; microcosm 2, -0.05; Table 2).

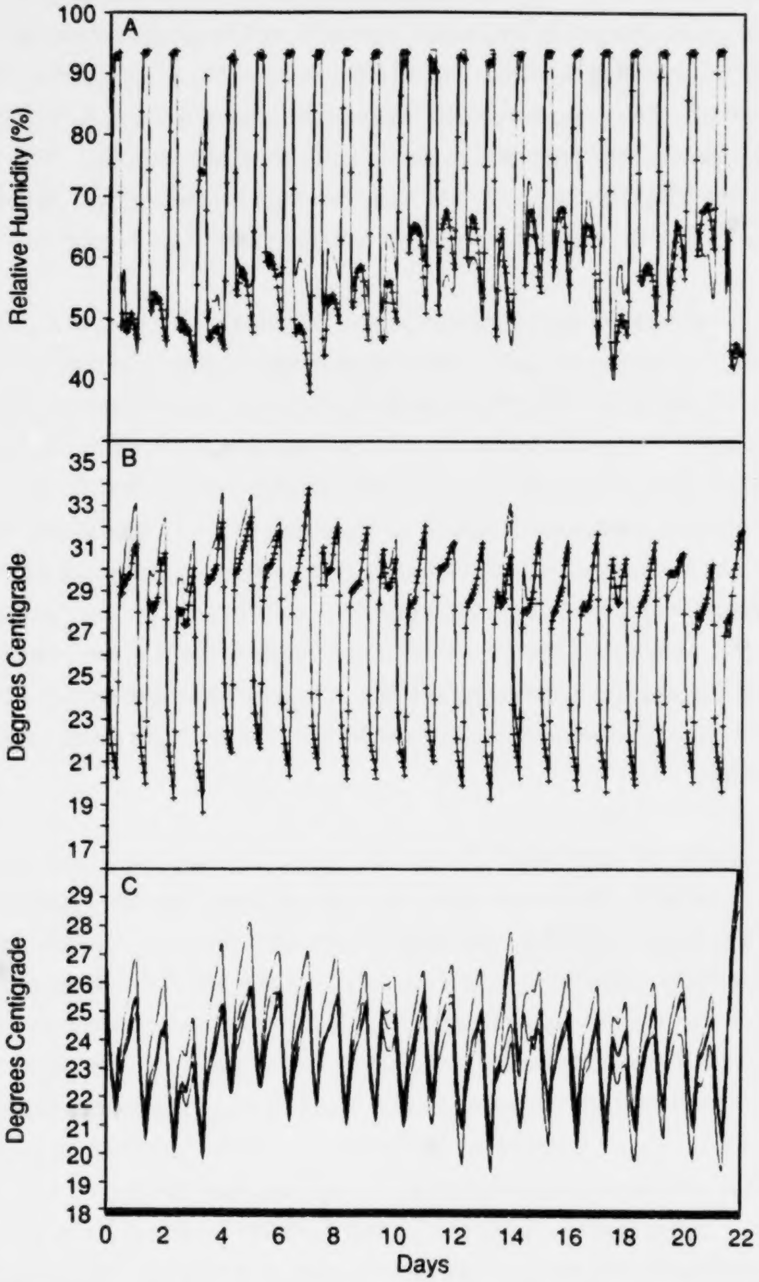

Figure 6. Environmental data from complex microcosms with similar environments (trial 1). A, % RH; B, air temperature. Symbols: -------, microcosm 1; +++++++, microcosm 2. C, soil temperature. The four lines in C represent a total of four temperature probes, two in each microcosm.

Table 1. Summary of Treatments and Environmental Data from Studies of *Enterobacter cloacae* (pBR322) Survival in Complex Microcosms

Trial Number	Microcosm Chamber	RH Conditions[a]	Hourly RH[b] Dark	Hourly RH[b] Light	Soil Moisture[c]	Hourly Air Temperature[d] Dark	Hourly Air Temperature[d] Light
1	1	High - Low	90 (6)[e]	57 (7)	73	22.1 (1.4)	30.0 (1.4)
	2	High - Low	88 (8)	55 (7)	60	21.8 (1.3)	29.6 (1.3)
2	1	High - Low	92 (4)	66 (6)	80	19.6 (1.7)	25.9 (2.0)
	2	High - Low	92 (4)	67 (7)	74	20.0 (1.6)	26.5 (1.8)
3	1	High - Low	88 (7)	50 (5)	77	18.7 (1.6)	28.4 (1.6)
	2	High - Low	87 (8)	48 (5)	71	19.8 (1.6)	28.5 (2.3)
4	1	High - Low	92 (4)	58 (6)	72	20.2 (1.0)	27.3 (0.8)
	2	Low	42 (3)	37 (3)	37	21.3 (0.9)	27.3 (0.7)
5	1	Low	40 (3)	36 (3)	43	20.3 (1.0)	26.4 (0.6)
	2	High - Low	87 (10)	46 (6)	60	19.2 (1.3)	27.1 (0.8)

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[a] High - Low, humidifier on during dark and off during light periods. Low, humidifier off during dark and light periods.

[b] Average of hourly RH readings during all dark or light periods for the duration of an experiment.

[c] Percent of the time during an experiment when the soil moisture content was between 62% and 100% of water-holding capacity.

[d] Average of hourly air temperature readings during all dark or light periods for the duration of an experiment.

[e] Standard deviation.

When analyses of variance were performed on plant or soil data from days 0, 5, 11, 15, and 21 of trials 1, 2, or 3, the "day effect" (i.e., variance about the mean of all plant or soil sample data from all days combined) was highly significant ($p \leq .005$) as the result of the marked difference between the day 0 value and those for days 5 through 21. This was confirmed by the same analyses with day 0 values omitted, which now showed the day effect to be insignificant ($p > .30$ for plant sample data; $p > .10$ for soil sample data). Table 3 summarizes the probabilities from the analyses done for the three pairwise combinations of the three trials, using plant and soil sample data from days 5 through 21. In all cases, no

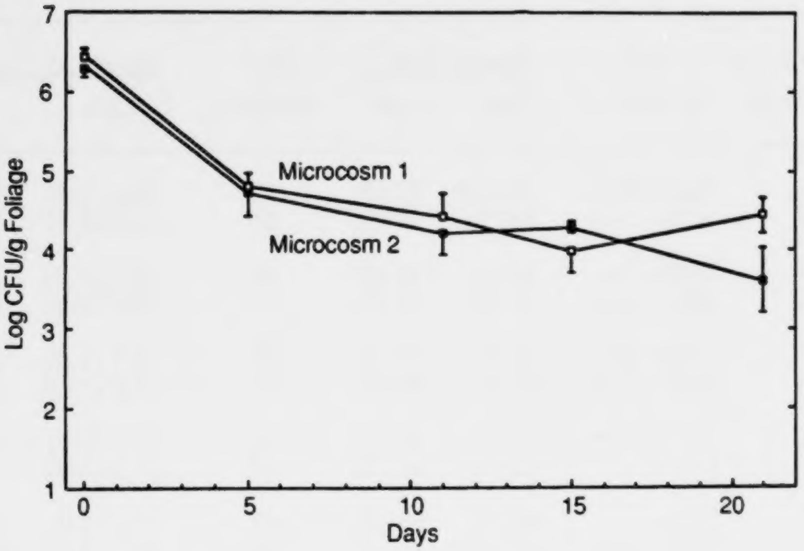

Figure 7. Survival of *Enterobacter cloacae* (pBR322) on bean leaves in complex microcosms with similar environments (trial 1).

differences between microcosms were observed at the $p = .05$ level. It is useful to compare the graphs from trials 1 and 2 (Figures 7 through 10) with the results of the statistical analyses (Table 3) to understand the relationship between the curves and the calculated probabilities.

In studies of the effects of environment on survival, statistical analyses were performed on two experiments that used a "cross-over" design; the treatment used in each microcosm during the first trial was applied to the other microcosm during the second trial. This reversal of treatments minimized possible experimental errors arising from chamber effects that were independent of the parameters being tested. With this experimental approach, the probability values from the three pairwise comparisons of trials 1 through 3 in Table 3 were used as indicators of the limits of resolution that had to be exceeded to distinguish differences between survival curves obtained from chambers with different treatments. This logic was applied in trials 4 and 5, which tested the influences of RH and soil moisture content on survival of *E. cloacae* (pBR322). RH was raised to about 90% during the dark period in one chamber, but maintained near 40% in the other chamber (Figure 11). It is apparent from the graph (Figure 12) and slopes (Table 1) that survival of the epiphytic population was not affected markedly by the RH treatment. However, survival in the soil (Figure 13; Table 2) was enhanced by

Table 2. Slopes of *Enterobacter cloacae* (pBR322) Survival Curves Using Complex Microcosms[a]

Trial Number	Microcosm Chamber	RH Conditions[b]	Foliar Populations Days 0-5	Foliar Populations Days 5-21	Soil Populations Days 0-5	Soil Populations Days 5-21
1	1	High - Low	-0.33a	-0.03	-0.16	-0.06
	2	High - Low	-0.32	-0.06	-0.21	-0.05
2	1	High - Low	-0.36	-0.04	-0.20	-0.04
	2	High - Low	-0.43	-0.02	-0.24	-0.05
3	1	High - Low	-0.35	-0.03	-0.12	-0.05
	2	High - Low	-0.50	-0.03	-0.24	-0.09
4	1	High - Low	-0.56	-0.05	-0.04	-0.10
	2	Low	-0.46	-0.06	-0.30	-0.01
5	1	Low	-0.44	-0.06	-0.33	-0.06
	2	High - Low	-0.57	-0.01	-0.14	-0.03

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[a] Log cfu/g per day.

[b] Environmental data in Table 1.

increased moisture content. These responses of the bacteria to the environmental treatments were verified by statistical analyses of populations presented in Table 3 (foliage, $p = .47$; $p = .003$).

Complex terrestrial microcosms, as is shown here, may serve as useful tools for preliminary assessments of viability of recombinant bacteria in lieu of field-based studies. Verification of their usefulness with parallel studies in the field is important, however. Further applications of the methods might include: a) long-term (3 months and longer) persistence of recombinant bacteria; b) effects of environment on ingestion, survival, and transport of bacteria in invertebrates and higher trophic levels; c) comparisons of GEM survival in soil and on plants in the presence and absence of selected invertebrate species; d) factors that affect bacterial colonization of leaf surfaces; and e) comparisons between survival of GEMs and the nonrecombinant parental strains.

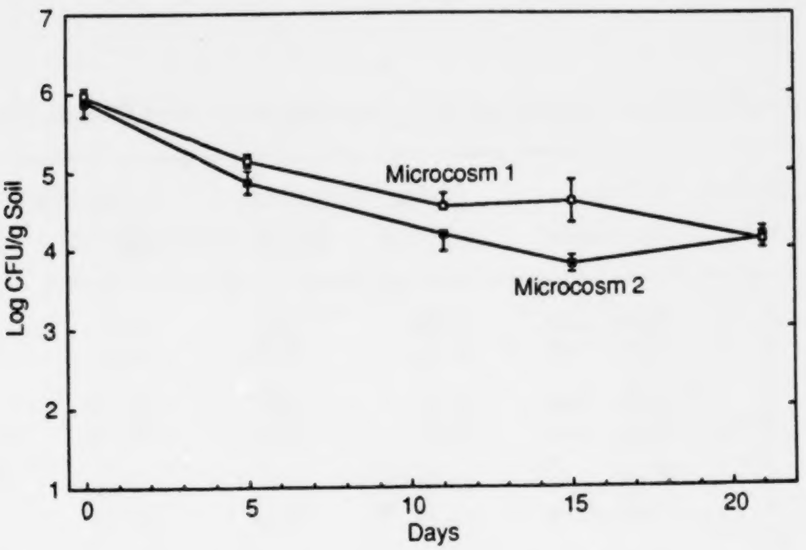

Figure 8. Survival of *Enterobacter cloacae* (pBR322) in soils in complex microcosms with similar environments (trial 1).

Table 3. Probabilities from Analyses of Variance to Determine the Effects of Treatment on Bacterial Survival[a]

Trials Compared[b]	Sample Type	Treatment Difference[c]	Probability
1 and 2	Foliage	None	0.50
	Soil	None	0.90
1 and 3	Foliage	None	0.53
	Soil	None	0.85
2 and 3	Foliage	None	0.23
	Soil	None	0.94
4 and 5	Foliage	Relative humidity	0.47
	Soil	Moisture content	0.003

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[a] Each trial consisted of a pair of microcosms inoculated with similar quantities of bacteria. Foliar and soil populations were enumerated on days 5, 11, 15, and 21 after spraying with *E. cloacae* (pBR322). Survival curves were compared to determine the similarity between pairwise combinations of experimental data.

[b] See Figures 7 through 10 for graphs of Trials 1 and 2. Trial 4 is shown in Figures 12 and 13.

[c] See Table 1 for environmental conditions.

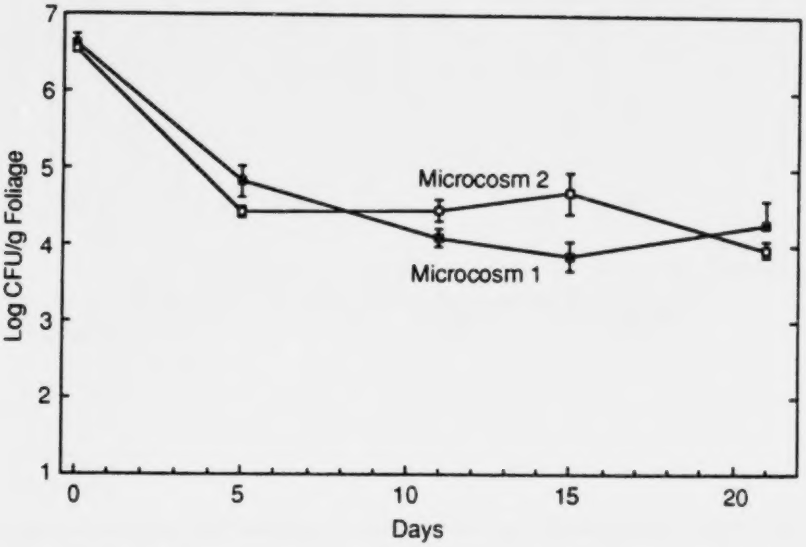

Figure 9. Survival of *Enterobacter cloacae* (pBR322) on bean leaves in complex microcosms with similar environments (trial 2).

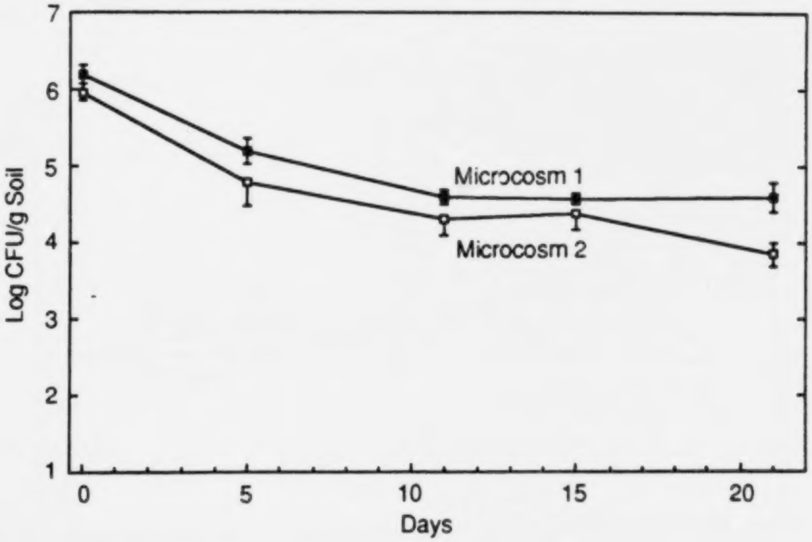

Figure 10. Survival of *Enterobacter cloacae* (pBR322) in soils in complex microcosms with similar environments (trial 2).

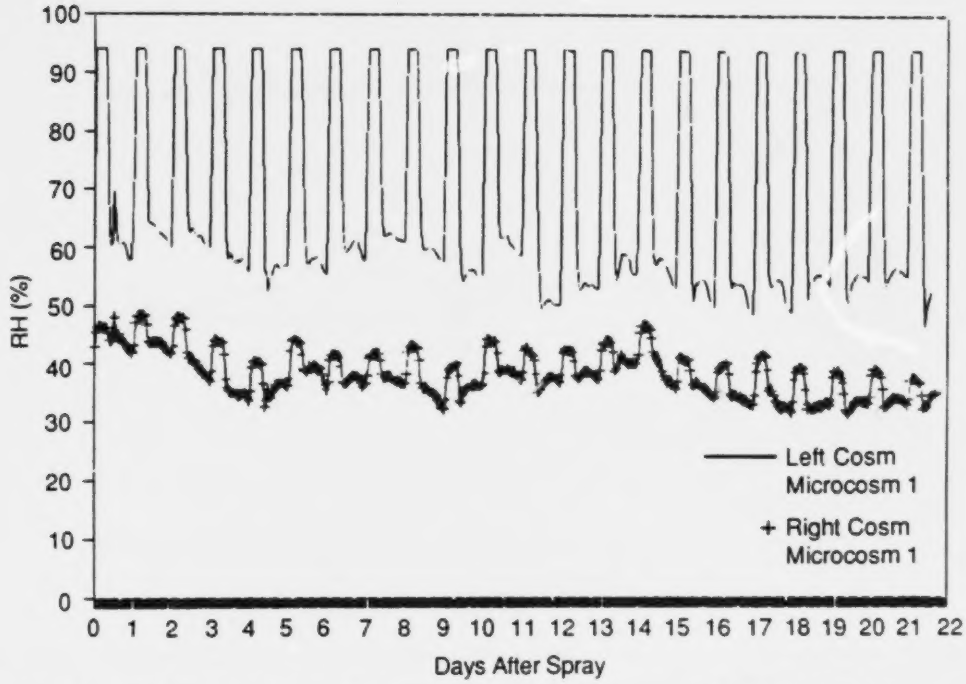

Figure 11. Environmental data from two complex microcosms with different RH treatments (trial 4). Symbols: -------, microcosm 1; +++++++, microcosm 2.

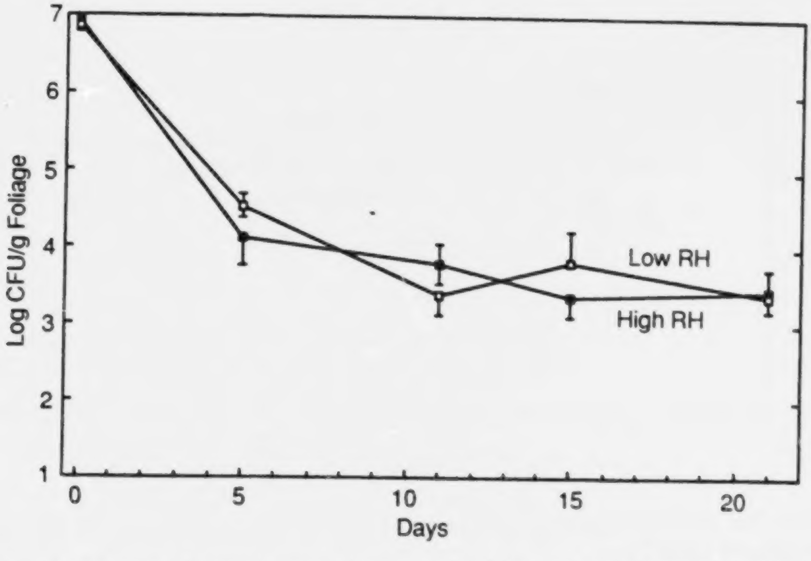

Figure 12. Survival of *Enterobacter cloacae* (pBR322) on bean leaves in complex microcosms with different RH treatments (trial 4).

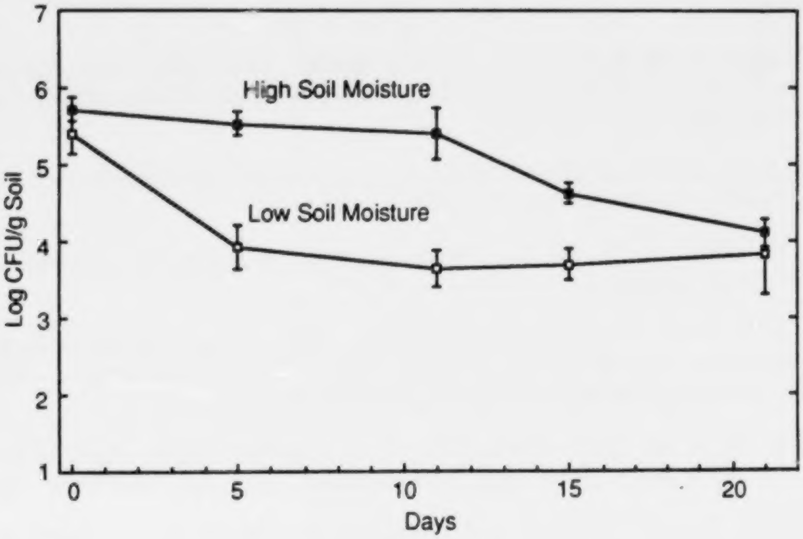

Figure 13. Survival of *Enterobacter cloacae* (pBR322) in soils in complex microcosms with different soil moisture contents (trial 4).

ACKNOWLEDGMENTS

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3.0 THE FATE OF RECOMBINANT PSEUDOMONADS IN MODIFIED SOIL-CORE MICROCOSMS (VERSACORES)

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INTRODUCTION

Microorganisms that have been genetically engineered to act as biological pesticides (19), frost retardants (13), or other agronomically useful products must be proven safe for release into the environment before they can be field tested. To achieve this objective, effective laboratory test methods must be developed that can assess likely behavior in the environment to determine the risk of releasing genetically engineered microorganisms. In addition, laboratory studies can provide information based on topics central to this issue such as survival and competitiveness of microorganisms in soil, and the effect of different parameters on organism fate such as roots, particle composition, water activity, and leaching.

Past studies on the fate of introduced organisms have usually used microorganisms that are spontaneously resistant to antibiotics (2,5,14,16). While this approach has provided much useful information, especially on *Rhizobium* ecology, it is often limited by a high background of antibiotic-resistant organisms in the indigenous soil population (12) and because the antibiotic resistance trait may not be ecologically neutral. Furthermore, questions unique to the issue of environmental use of genetically engineered organisms, such as the fate of the engineered gene, must be answered in a test microcosm. The engineered strains that have been studied in soil have been labeled with a plasmid or transposon that carries drug-resistant markers (18) or the lactose utilization marker (4). These engineered strains have only been monitored in soil by selective plating procedures. Bacteria often become debilitated after incubation in soil and are unable to grow on selective media (3), and this may result in an underestimation in the survival of the trait or organism in nature.

We have developed an alternative method for detection of genetically engineered microorganisms in soil (8) that relies on extraction of total soil bacterial DNA. The isolated DNA can then be analyzed using gene probes specific for the added microorganisms. This methodology allowed us to detect engineered *Bradyrhizobium* (8) and *Pseudomonas* strains (see Section 5.2) in soil. The organisms were engineered by insertion of a specific gene, *nptII*, which confers kanamycin resistance, into the chromosomes. This gene was found to have a low background hybridization in the indigenous soil bacterial community. The recombinant organisms could then be traced in soil using the *nptII* gene as a specific probe.

The soil core represents the most convenient, realistic microcosm of the soil microorganism environment, and is thus the most logical approach for establishing a method to assess the fate and behavior of genetically engineered microorganisms. The core maintains the natural nutrient supply and the physical relationships, including competition, among indigenous organisms. It can also support plants, is easily used in rainfall and leaching studies, and is convenient for use in the laboratory. Thus, the objective of this study was to refine and test a soil-core system for studying the fate of introduced genetically engineered microorganisms. Because of this versatility of this core, designed to allow several modes of organism introduction as well as analysis, we have named it "Versacore."

Pseudomonas species were chosen as the engineered organisms used to evaluate the Versacore design because of their importance in agronomic and biodegradation applications. *Pseudomonas cepacia* PCO1224 and PCO1215 (R388::Tn*904*) are being considered by the U.S. Environmental Protection Agency (EPA) as potential benchmark strains for evaluating and standardizing assessment methodology. *Pseudomonas* sp. B8 is an aggressive root-colonizing, toxin-producing microorganism that inhibits growth of winter wheat (6). Engineered derivatives of *Pseudomonas cepacia* PCO1224 and *Pseudomonas* sp. B8, in addition to the plasmid-containing strain, *P. cepacia* PCO1215 (R388::Tn*904*), were used as model organisms in this study. The Versacore was used to evaluate survival and dissemination of recombinant pseudomonads from the point of inoculation to different depths of the core in the presence and absence of plants.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The three bacterial strains used in this study were genetically engineered species of *Pseudomonas*. Two strains, *Pseudomonas cepacia* PCO1224 (obtained from Ron Olsen, University of Michigan, East Lansing, Michigan) and *Pseudomonas* sp. B8 (obtained from Lloyd Elliott, Washington State University, Pullman, Washington), were engineered by inserting into their chromosomes the *nptII* gene, which confers kanamycin resistance (see Section 5.2). The resulting recombinants, *P. cepacia* PCO1224-1 (PC-1) and *Pseudomonas* sp. B8-1 (B8-1), were resistant to high concentrations of kanamycin (500 µg/ml).

The pseudomonads were cultivated on Kings B (10) liquid or solid media. *P. cepacia* PCO1224 and *Pseudomonas* sp. B8 are naturally resistant to nalidixic acid or rifampicin, respectively. Antibiotics when used for solid media were at the following concentrations: nalidixic acid, 400 µg/ml; rifampicin, 200 µg/ml; and kanamycin, 500 µg/ml. *Pseudomonas* sp. B8 and the recombinant derivative B8-1 were cultivated at 25°C, whereas *Pseudomonas cepacia* PCO1224 and the recombinant derivative PC-1 were grown at 37°C. When the two strains were added together or inoculated into soil, the incubation was at 25°C. The two recombinant strains could be distinguished from each other on the basis of colony color: PC-1 formed yellow colonies whereas B8-1 formed white colonies on Kings B. Furthermore, the strains could be distinguished on different agar media by the differences in their antibiotic resistances.

An additional strain, *Pseudomonas cepacia* PCO1215 (R388::Tn*904*) (obtained from Ron Olsen, University of Michigan) harbors the engineered plasmid R388::Tn*904* containing trimethoprim- and streptomycin resistance determinants. This strain was selectively isolated on solid minimal medium containing trimethoprim (200 µg/ml), streptomycin (2000 µg/ml), and proline (50 µg/ml). Cycloheximide (200 µg/ml) was added to all solid media used for selection of the recombinant strains from the soil population to reduce fungal background.

Versacore Microcosm Design

The Versacore microcosm design is diagramatically represented in Figure 1. Versacores were constructed from clear cast acrylic tubing (AIN Plastics, Southfield, Michigan) with an outer diameter of 9.5 cm and a wall thickness of 0.48 cm. The cylinders were cut into 2.5-cm sections and reassembled to the desired height by taping them together with waterproof tape.

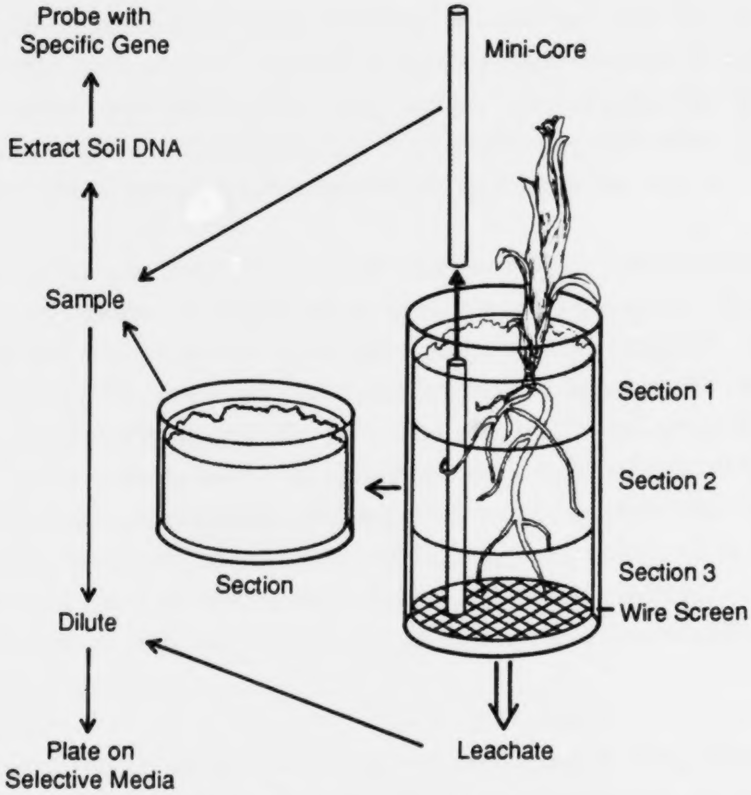
Probe with Specific Gene
Mini-Core
Extract Soil DNA
Sample
Section 1
Section 2
Section
Section 3
Wire Screen
Dilute
Leachate
Plate on Selective Media

Figure 1. Versacore design.

For the experiments used in this study, three sections were taped together. A wire mesh (1.5-mm openings) was glued between the bottom of the third section and an additional fourth section using methylene chloride as the cementing agent.

Capac loam soil (fine-loamy, mixed, mesic Aeric Ochraqualf) was sieved through a 2-mm sieve; 550 g was added to the core using a wide-mouth funnel, starting from the edges and filling in toward the center. The soil was packed by tapping the core on the bench top as the soil was added to achieve a bulk density of approximately 1.3 g/cm^3. The cores were brought to field capacity (approximately 23% moisture) by setting them in a pan of distilled water overnight. By morning, the water had migrated to the surface. The cores were then removed from the water, allowed to drain, and covered at both ends with Parafilm.

Cores were planted with five wheat seeds or three corn seeds per core. Corn seeds were pretreated with captan and methoxychlor, and wheat seeds were rinsed in 1.5% sodium hypochlorite, followed by a wash in filter-sterilized distilled water. The seeds were added to the soil at the top of the cores using flame-sterilized forceps.

The cores were incubated under constant temperature and humidity conditions (25°C, 52% humidity) in an incubator room. Constant light was provided by fluorescent lights (intensity, 26 microeinsteins/s^2). After plant germination, the Parafilm was removed from the top of the cores, and the moisture content of the soil was maintained by weighing the cores at daily intervals and adding water equal to the amount lost.

Bacterial Inoculation

Cells were grown in liquid media to an optical density (OD; 640 nm) of approximately 1.0, harvested by centrifugation, and washed in 10 m*M* sodium phosphate buffer (pH 7.0). The cells were then starved for 2 to 6 h in the phosphate buffer. If necessary, the suspension was centrifuged 10 min at 8000 x *g* and resuspended to the desired concentration. In most experiments, the cell suspension was added to the top of the core using a 10-ml syringe with an 18-gauge needle. Care was taken to distribute the inoculum slowly and evenly over the surface. In some experiments the inoculum was thoroughly mixed throughout the soil before the soil was packed into the core. In these experiments the inoculum was again added using a 10-ml syringe and the soil was continually mixed as the inoculum was added.

For the preemptive colonization experiments, one *Pseudomonas* strain was mixed throughout the soil, the soil was packed into cores, and the cores were planted with wheat. After 1 week incubation, the other strain was added to the surface of the cores and the cores were incubated an additional 9 days and sampled every 3 days with mini-cores. As controls, both pseudomonads were also added together either to the top or throughout the soil. Additional controls included cores to which each organism was added singly to the top or was singly mixed throughout the soil. Phosphate buffer was added to controls not receiving a microbial inoculum.

Mini-Core Sampling

Cores were periodically sampled by taking mini-cores throughout the length of the core using a flame-sterilized core borer (9-mm diameter). The holes left in the Versacore were filled with sterile inverted Pasteur pipets that fit firmly in the holes. Two different samples were taken per core at each sampling time and combined in a weighing dish; 1 g of the sample was used to enumerate the bacteria added. The sample was added to 9.5 ml sodium pyrophosphate (0.1%), sodium chloride (0.9%), and Tween 80 (0.1%), and vortexed vigorously for 1 min. The sample was then serially diluted (1:10) in 9 ml of 10 m*M* sodium phosphate buffer (pH 7.0). The remainder of the sample was used for measurement of soil moisture content. All counts on agar media were converted to log CFU per gram (dry weight) of soil.

Sampling by Leaching

For experiments where the cores were sampled by leaching, the cores were set into individual holes of a wooden platform. A funnel was placed underneath the core and into the mouth of a preweighed 250-ml centrifuge bottle. The cores were leached with 200 ml nonsterile tap water (equivalent to 1 in. of rainfall) using a 50-ml syringe, taking care to prevent the water from draining down the sides of the cylinder. The leachate was collected and the bottle reweighed to determine the amount of water that drained through the soil column. The leachate was centrifuged 15 min at 23,000 x *g* to concentrate the cells. The cell pellet was resuspended in 10 ml of 50 m*M* Tris buffer (pH 7.0), and serially diluted (1:10) in Tris buffer. Dilutions were plated onto solid medium, and counts were converted to a per-milliliter-leachate basis.

Sampling by Sectioning

Cores were divided into sections by removal of the waterproof tape. The soil core was cut between sections using a flat spatula prewashed in 10% sodium hypochlorite and rinsed with distilled water. The spatula was rewashed between each slice to avoid contamination. Each slice of soil (± roots) was mixed on an individual sheet of aluminum foil. A 50-g sample was taken for extraction of total soil bacterial DNA. A 10-g sample of the soil was used for enumeration by selective plating. An additional soil sample was taken for soil dry weight measurement. Cores sampled by sectioning were not leached before dismantling in these experiments to avoid movement of bacteria to lower layers with the large volume of water. Instead, only small amounts of water were added to maintain soil moisture.

Rhizosphere and Rhizoplane

The root populations of the recombinant strains in Versacores planted with wheat or corn were determined essentially as described by Fredrickson and Elliott (6). After sacrificing the cores, the roots were collected and gently shaken in 50 ml of buffer consisting of sodium pyrophosphate (1 g/liter), sodium chloride (9 g/liter), and Tween 80 (0.1%) in Erlenmeyer flasks. The rhizosphere population was determined by shaking the soil in the flask for 1 h, then serially diluting and plating onto selective media. The soil was collected by vacuum filtration and dried to determine the dry weight of the rhizosphere soil. Rhizoplane populations were enumerated by shaking the roots in 50 ml of the buffer just described for 1 h, then plating dilutions onto selective media. The roots were collected for dry weight determination. Rhizosphere or rhizoplane counts were expressed as log CFU per gram dry weight of soil or per gram dry weight of root, respectively.

Soil DNA Extraction and Hybridization

Soil DNA was extracted by our previously described method (8). Briefly, a portion of the soil bacterial community was eluted from soil by repeated cycles of blending and differential centrifugation. The bacterial cell pellet was lysed, and the DNA was separated, concentrated, and purified by ultracentrifugation in a CsCl-ethidium bromide density gradient (15).

The isolated soil DNA was analyzed by slot blot hybridization (9). A known concentration of DNA was added to nitrocellose through individual wells of a slot blot manifold (Bethesda Research Laboratories, Rockville, Maryland). The DNA was first denatured with 1/10 volume

3 *N* NaOH and heated 5 min at 100°C, then neutralized with 1 volume of 2 *N* ammonium acetate on ice, and added to a nitrocellulose filter equilibrated with 1 *M* ammonium acetate using the slot blot aparatus. After vacuum filtration of DNA onto the membrane filter, the filter was air dried and baked 2 h at 80°C. Prehybridization was carried out in bags containing approximately 15 ml prehybridization solution with 50% formamide at 42°C for 12 to 24 h. After prehybridization, single-stranded probe was prepared using a M13 phage vector as we have previously described (8). Alternatively, if sensitivity was not a critical issue, the *nptII* probe was prepared by nick translation of the plasmid pKC7, which contains the *nptII* gene (1). The R388::Tn*904* plasmid was also labeled by nick translation. Hybridization was then conducted for an additional 12 to 24 h at 42°C. After hybridization, the nitrocellulose membrane was washed under stringent conditions to remove unbound probe as follows: briefly in 6x sodium chloride:sodium citrate (SSC) + 0.1% sodium dodecyl sulfate (SDS) at room temperature, 15 min each at room temperature in 2x SSC + 0.1% SDS, 0.5x SSC + 0.1% SDS, and 0.1x SSC + 0.1% SDS, respectively, followed by 30 min at 50°C in 0.1x SSC + 1% SDS. The filter was examined for bound sequences by autoradiography using Dupont Cronex 4 medical x-ray film.

Southern blot analysis was conducted by standard methodology (17). DNA isolated from soil was digested with the restriction enzyme *Cla*I. The DNA fragments were separated on a 1% agarose gel and transferred to a nitrocellulose filter. The filter was prehybridized and hybridized as described for slot blots, with the addition of 5% dextran sulfate in the hybridization solution.

RESULTS

Survival of Genetically Engineered Pseudomonads in Soil Cores

The fates of the three engineered strains in the Versacores as determined by plate counts are shown as follows: Figure 2, *P. cepacia* PCO1215 engineered by plasmid transformation with R388::Tn*904*; Figure 3, *P. cepacia* PC-1 (with *nptII*); and Figure 4, *Pseudomonas* sp. B8-1 (with *nptII*). Minicore sampling showed that all these strains decreased to the range of 10^5 organisms/g soil after 10 to 14 days, with PC-1 maintaining the highest density perhaps because its larger inoculum (Figure 3). The presence of corn roots did not have any significant effect on the soil populations of either PC-1 or B8-1 (Figures 3 and 4).

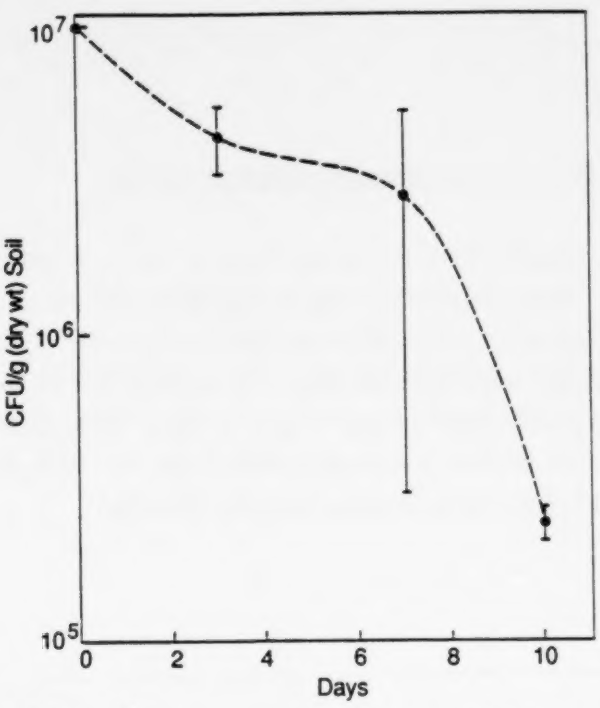

Figure 2. Survival of *Pseudomonas cepacia* PCO1215 (R388::Tn*904*) in Versacores planted with corn. Counts represent mean of three replicate cores.

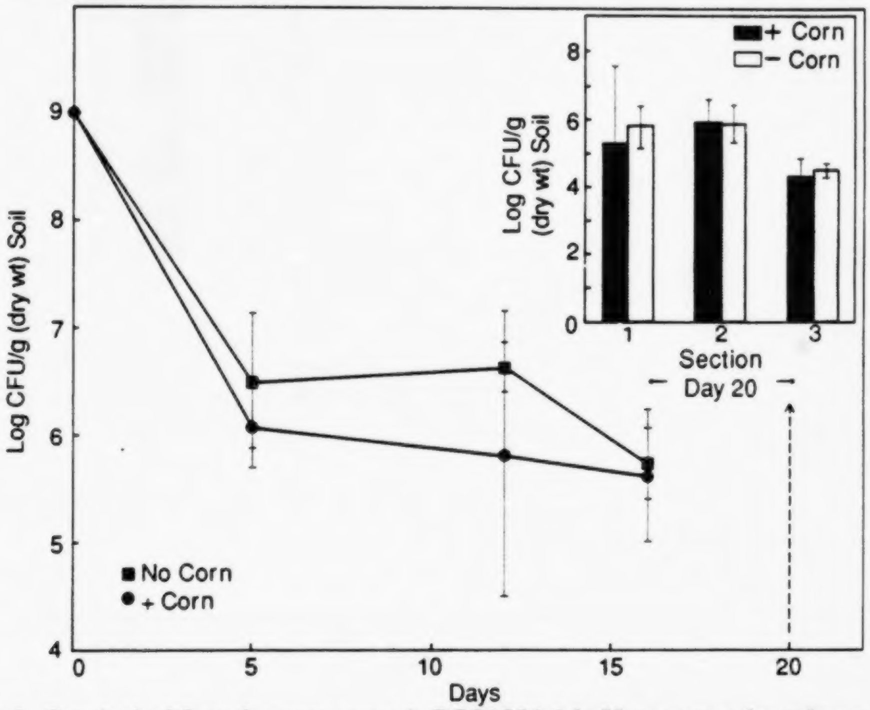

Figure 3. Survival of *Pseudomonas cepacia* PCO1224-1 in Versacores planted or not planted with corn. Mini-core samples were taken until day 17 of incubation. After 20 days, cores were divided into three 1-in. sections, and cells at each depth were enumerated on selective medium. Counts represent mean of three replicate cores.

Movement of Genetically Engineered Pseudomanads in Soil Cores

Versacores were sampled in a variety of ways to assay movement of engineered pseudomonads in soil. These included leaching of bacteria through the soil profile with water and examination of bacterial density at different depths by selective plating and gene probe methodologies. Versacores inoculated with B8-1 were leached after 29 days incubation, and the leachate was plated onto selective medium (Figure 4, insert). When the cores were leached, the number of cells in the leachate was higher for the cores that were planted, presumably because of the enhanced movement of bacteria along root channels.

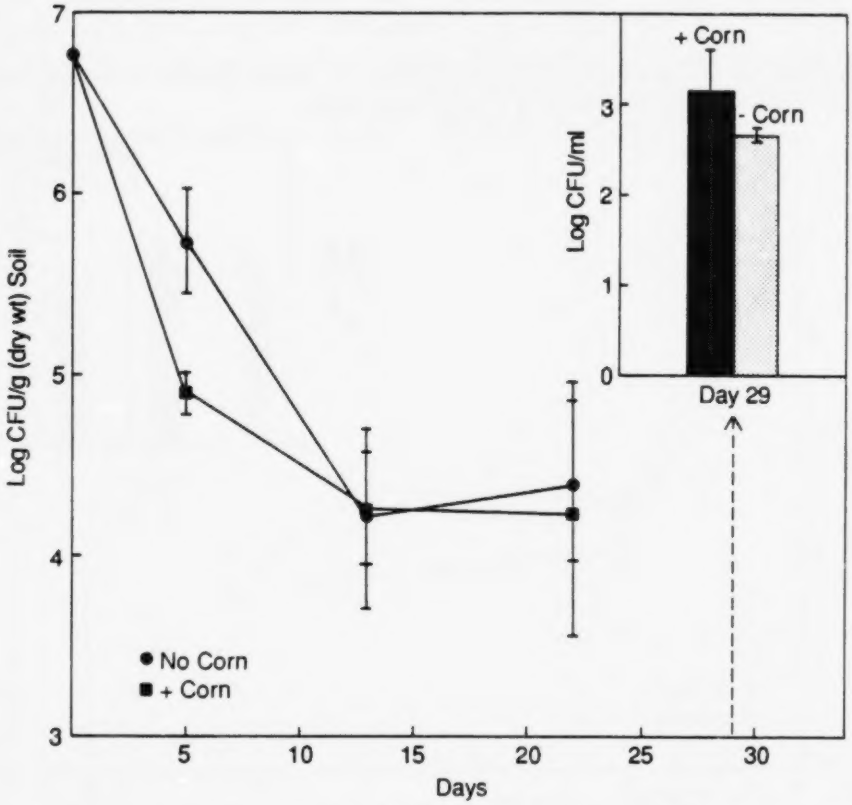

Figure 4. Survival of *Pseudomonas* sp. B8-1 in Versacores planted or not planted with corn. Mini-core samples were taken until day 22 of incubation. After 29 days the cores were leached with the equivalent of 1 in. of rainfall and the leachate was plated onto selective medium. Counts represent means of three replicate cores.

In a separate experiment after 20 days incubation, the cores were not leached but the density of PC-1 cells at different depths in the soil cores was determined by separating the cores into three sections and plating samples from each section on selective medium. The data indicate that PC-1 maintained a high population at the top of the core (the point of addition) and in the middle core section. The numbers of PC-1 in the bottom section was significantly lower, but cells were still detected at this depth. When sampling by sectioning, we did not find a significant difference between planted and unplanted cores, indicating that roots had no apparent effect on movement through this nonleached soil. The amount of water added to the cores to maintain the moisture content were very small compared to the 200 ml used to leach the cores in the previous experiment, which is a likely explanation for the observed differences in bacterial movement when this experiment is compared to the previous one.

Gene probes were employed as another method to follow movement of bacteria in soil. The single-stranded *nptII* gene probe was used to detect PC-1 and B8-1. The intensity of hybridization of the probe increased with an increase in the concentration of the cells added to soil (Figure 5). Versacores inoculated with PC-1 or B8-1 and incubated for 7 days were then dissassembled into the three sections. DNA extracted from each section was probed with the *nptII* gene (Figure 6). The highest number of cells were detected in the upper section of soil corresponding with plate count data for the two organisms (Table 1).

Gene probes were also used to trace the plasmid-containing strain *P. cepacia* PCO1215 at different depths in the soil core. A slot blot of DNA extracted from soil at the end of the incubation period for the experiment reported in Figure 2 is shown in Figure 7. The isolated DNA was probed with the R388::Tn*904* plasmid. The greatest intensity of hybridization was to DNA isolated from the upper layer of the cores. There was some background hybridization to the control probed with R388::Tn*904*, possibly because a plasmid probe that might have homologous sequences, such as replication regions, was used in the indigenous bacterial community. Previous results (8) indicated that a nonindigenous gene, *nptII*, had negligible background in the soil community.

Effect of Co-Inoculation on the Fate of Two Recombinant Pseudomonads in Soil

The two pseudomonads were added to the same soil core to determine the effect of co-inoculation and movement on survival. Plate counts taken over time are presented in Table 2. The two organisms were distinguished from each other using two different selective media.

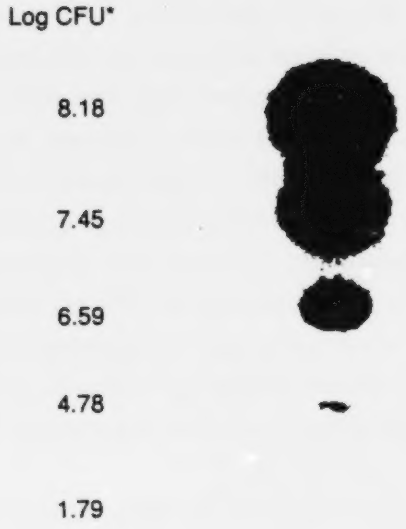

Figure 5. Slot blot of DNA isolated from soil samples inoculated with different concentrations of B8-1. The same amount (10 µg) of DNA was added to each slot; thus, differences in hybridization intensity resulted from differences in *nptII* gene concentration in soil samples. Cell density was verified by selective plating at time of extraction. Log CFU was calculated per gram dry weight of soil (*).

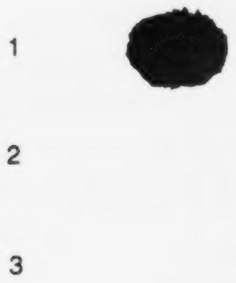

Figure 6. Slot blot of DNA isolated from different depths of soil in Versacores inoculated with PC-1. The same amount of DNA was added to individual slots on filter, and blot was probed with single-stranded *nptII*. The numerals 1, 2, and 3 refer to soil-core depths of 2.5, 5.0, and 7.5, respectively. Similar results were obtained for B8-1.

Table 1. Log CFU per Gram (dry weight) of Soil of Two Recombinant *Pseudomonas* Strains at Three Depths After 8 Days of Incubation in Versacores Planted with Wheat[a]

Depth (cm)	B8-1	PC-1
2.5	5.93	7.83
5.0	2.55	4.84
7.5	1.14	0.89

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[a] Counts represent the mean of three replicate cores.

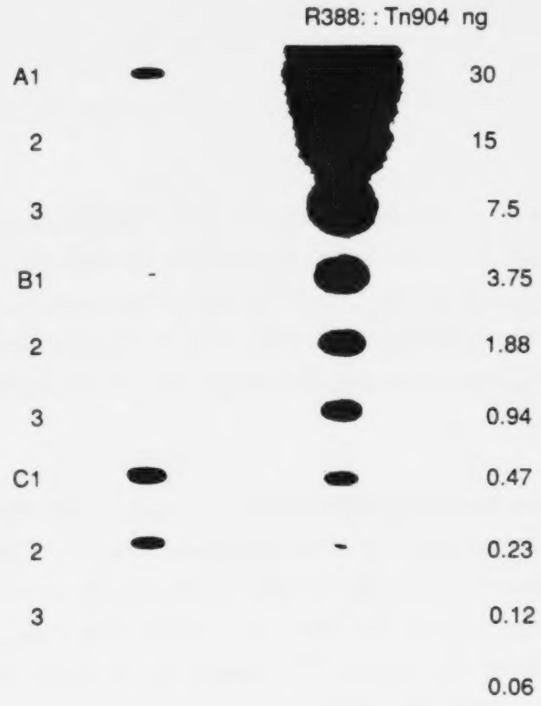

Figure 7. Slot blot isolated from three depths of soil cores inoculated with *Pseudomonas cepacia* PCO1215 (R388::Tn*904*). Blot was probed with [^{32}P]-labeled R388::Tn*904*). A and C, samples inoculated with *P. cepacia* strain; B is uninoculated control. The numerals 1, 2, and 3 refer to soil-core depths of 2.5, 5.0, and 7.5, respectively. Serial 1:2 dilutions of purified R388::Tn*904* DNA are given for comparison.

Table 2. Enumeration of PC-1 and B8-1 Added Together to Soil Cores Planted or Not Planted with Wheat

Time		Log CFU per Gram (dry weight) Soil[a,b]					
		B8-1			PC-1		
Wheat[c]	Days	2.5	5.0	7.5	2.5	5.0	7.5
+	0.5	7.83	<2	<1	8.52	4.92	<1
+	4	6.37	<2	<1	7.92	4.63	<1
+	18	4.42	2.73	1.72	6.05	4.33	0.91
-	18	4.64	<2	3.17	5.91	2.32	0.29

---

[a] At depths of 2.5, 5.0, and 7.5 cm from soil surface.

[b] Counts represent the mean of three replicate cores.

[c] Wheat planted, +; no wheat, -.

DNA was extracted at the first two sampling dates to detect the presence of the added organisms by slot blot analysis (Figure 8). Duplicate samples from 0.5 and 4 days were analyzed using the single-stranded *npt*II gene as a probe. Comparison of intensity indicated that the greatest numbers of the added recombinant pseudomonads were in the uppermost layers of the soil (the point of inoculum). The plate count data did not correlate to the hybridization results at the second sampling date.

PC-1 and B8-1 cannot be distinguished from each other by slot blot analysis when added together because they are engineered with the same gene insertion. Therefore, we cannot tell how much of the hybridization in Figure 8 resulted from each strain. However, the strains can be distinguished from each other in the same soil sample by agarose gel separation of *Cla*I restriction fragments followed by Southern blot analysis, because the *Cla*I fragment containing the *nptII* is a different size for the two strains (see Section 2.0).

The two *Pseudomonas* strains were added together to soil to determine the effect of co-inoculation on colonization of wheat roots. *Pseudomonas* sp. B8 is known to be an aggressive

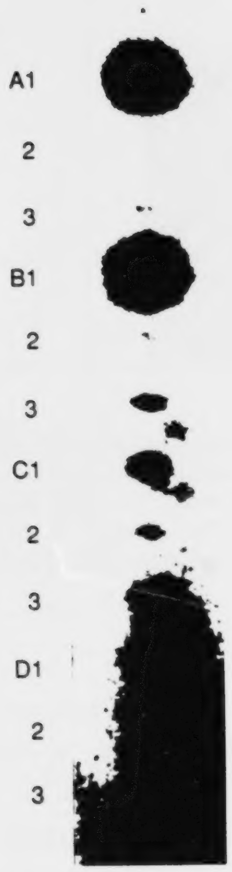

Figure 8. Slot blot of DNA isolated from different depths of soil in Versacores inoculated with both PC-1 and B8-1. The same amount of DNA (10 μg) was loaded into each slot, and blot was probed with single-stranded *nptII*. (A) and (B) were isolated from duplicate cores after 0.5 days; (C) and (D) were isolated from duplicate cores after 4 days of incubation. The numerals 1, 2, and 3 refer to soil-core depths of 2.5, 5.0, and 7.5, respectively.

root colonizer (6). When the engineered strain was compared to the wild-type organism, they did not differ in root-colonizing ability (Figure 9).

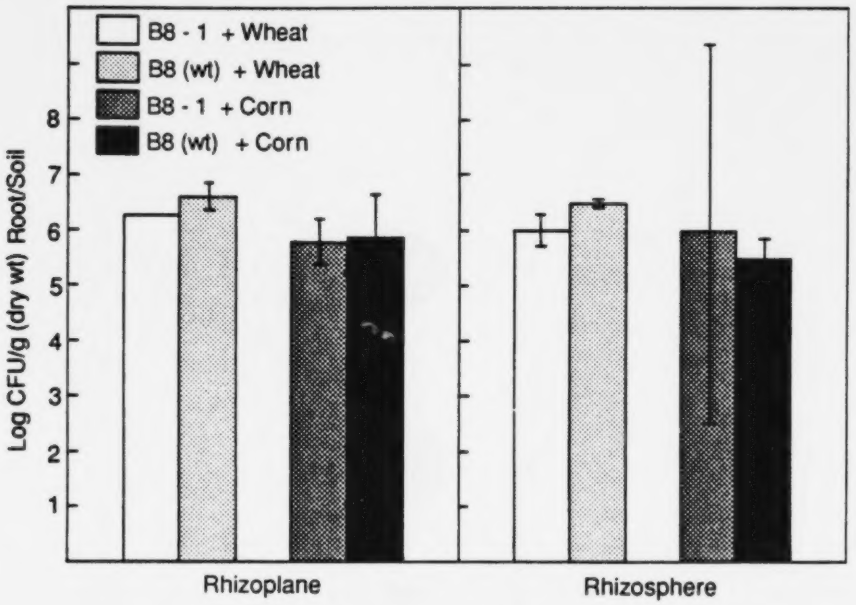

Figure 9. Comparison of *Pseudomonas* sp. B8 [wild type (wt)] and *Pseudomonas* sp. B8-1 (engineered derivative) as to root-colonizing ability on wheat or corn. Small cores were packed with 40 g Capac soil and planted with wheat or corn or left unplanted. Duplicate cores were inoculated with either B8 or B8-1 (10^7 cells/g soil). After 9 days of incubation, roots were removed from cores, and rhizosphere and rhizoplane populations were enumerated on selective media.

The known root colonizer, B8-1, was then compared to PC-1 for root colonization ability (Table 3). The two strains were added together or separately to soil planted with wheat. Samples were taken from the rhizosphere, rhizoplane, and leachate after 2 weeks, and analyzed by selective plating. Interestingly, the two microorganisms showed similar root colonization ability. In addition, there did not appear to be a competitive effect when the two organisms were co-inoculated into the same cores.

The effect of the method of co-inoculation (i.e., top or throughout) on subsequent root colonization of the two strains was determined (Table 4). There was no significant difference in the survival of either strain by these different inoculation methods (Figure 10). At the end of the experiment the cores were sacrificed, and the rhizosphere, rhizoplane, and soil bacterial populations were enumerated on selective media (Table 4). B8-1 counts were the same for both inoculation methods. PC-1 had higher counts when added to the top than when added throughout the soil, and the soil population of PC-1 was higher than that of B8-1.

Table 3. Population Densities of Two Recombinant *Pseudomonas* Species in Versacores With and Without Wheat Roots[a]

		Log CFU[b]		
Treatment	Organism	Rhizosphere	Rhizoplane	Leachate
1	B8-1	7.2	7.1	5.5
2	B8-1	7.0	7.2	6.3
	PC-1	6.8	7.0	5.5
3	PC-1	7.2	7.8	3.0
4	B8-1	--	--	6.2
	PC-1	--	--	6.8

---

[a] Treatments 1, 2, and 3 were planted with wheat; treatment 4 was not planted. B8-1 was added alone in treatment 1, and PC-1 alone in treatment 3. Both PC-1 and B8-1 were added in treatments 2 and 4. Counts represent the mean of triplicate cores.

[b] Per gram soil, per gram roots, or per milliliter leachate, respectively.

Table 4. Rhizosphere, Rhizoplane, and Bulk Soil Populations of PC-1 and B8-1 in Versacores[a]

	Log CFU per Gram (dry weight) Root or Soil[b]			
	Top		Mixed Throughout	
Sample	PC-1	B8-1	PC-1	B8-1
Rhizosphere	3.7	4.2	2.6	3.6
Rhizoplane	3.9	4.2	1.8	4.0
Soil	5.6	3	5.3	4.2

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[a] Incubation time was 3 weeks after addition of strains if added throughout, and 2 weeks if added to the top (see Figure 10).

[b] Counts represent the mean of three replicate cores.

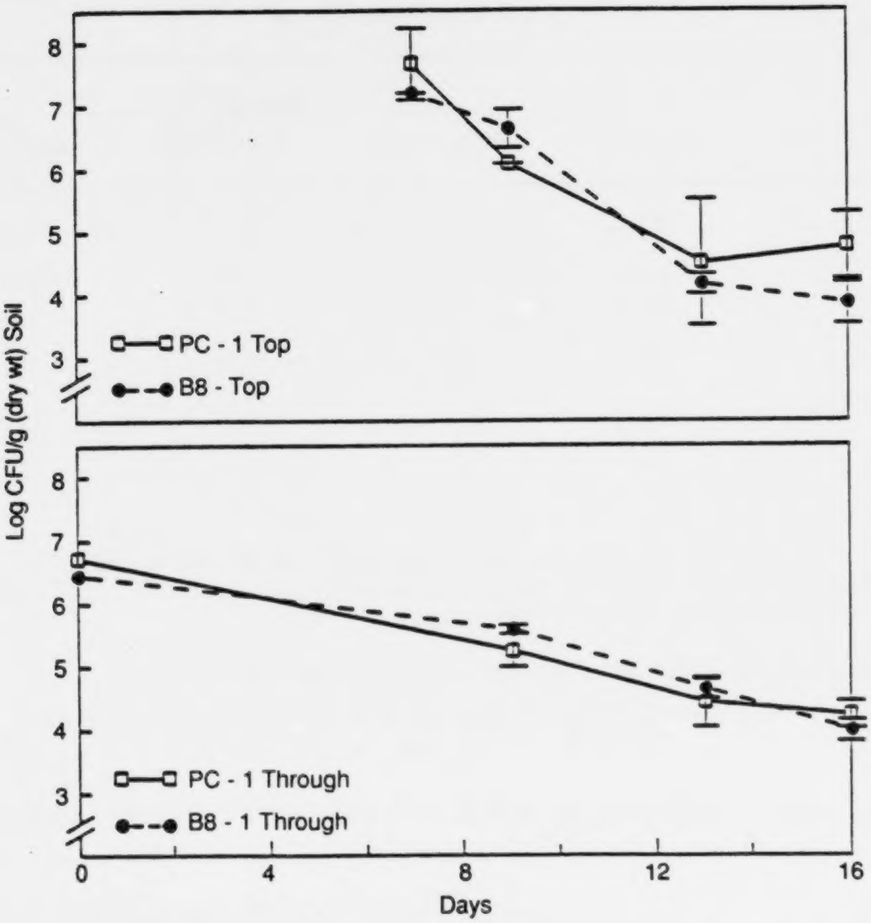

Figure 10. Survival of Pc-1 and B8-1 added together to top of core or mixed throughout soil in planted Versacores. Counts represent mean of three replicate cores.

Effect of Preemptive Colonization on Survival

Because the PC-1 and B8-1 strains appeared to be similar in their root colonization ability, we investigated the effect of preemptive colonization by one of the strains on establishment of the second, compared to controls with just one organism added. When either strain was allowed to colonize alone for 7 days and then the second strain was added, there was no noticeable interference on colonization of the second strain by the prior establishment to the first when compared to controls (Figure 11).

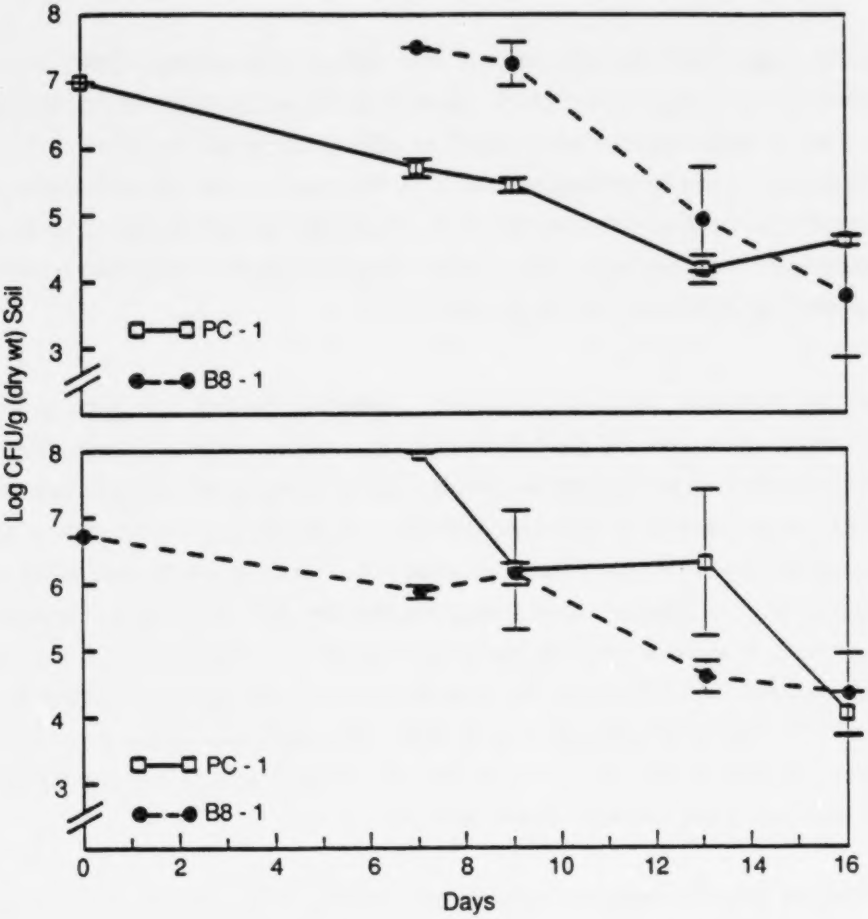

Figure 11. Establishment and subsequent colonization and survival of PC-1 and B8-1 added consecutively to soil in Versacores planted with wheat. At day 0, strains were added by mixing throughout soil and cores were planted. After 7 days of incubation, the second strain was added to surface of soil core. Counts represent mean of three replicate cores.

DISCUSSION

A versatile soil-core design was successfully used to monitor the fate and competitiveness of genetically engineered microorganisms introduced into a natural soil community. The design allowed temporal sampling by extraction of mini-cores from the larger core, assessment of movement and leaching of the added organisms, and determination of the effect of roots on colonization and movement. Both conventional selective plate counting and gene probe methodologies were conveniently used to assess the fate of singly added organisms or to distinguish between two cointroduced organisms. These methods were used to trace genetically engineered bacteria labeled with either chromosomal DNA insertions or autonomously replicating plasmid DNA. In other studies we have shown that this design can also be used to study recombination in soil by adding donor and recipient strains in soil to different sections either by mixing into soil or by injecting into the soil from a side port in the core (Dwyer et al., personal communication). Thus, this versatile design is both convenient and appropriate for evaluating a number of experimental questions important to assessing the fate of genetically engineered microorganisms in soil.

The two detection methods employed - selective plating and gene probe - have complementary advantages and disadvantages in these studies. Selective plating is easier, more sensitive, and offers more capacity for sample number. Gene probe methods, however, detect organisms that are stressed or otherwise nonculturable on selective media, and can also detect rearrangements or exchanges of the target gene (8). Slot blots are the method of choice for analysis of a large number of samples because they are easy to prepare. However, if two organisms such as the two pseudomonads in our study are engineered with the same DNA insertion, they cannot be distinguished from each other in the same soil sample by slot blot analysis. They can be distinguished by Southern blot analysis, but the latter is more time consuming and slightly less sensitive. On the other hand, it usually reduces any nonspecific background binding that can occur in slot blots (8).

Using these detection methods, Versacores were sampled in a variety of ways to determine the fate of the recombinant pseudomonads in soil. We did not find an effect of roots on survival or movement except when the cores were leached (see Figure 4). In this case greater numbers were obtained in the leachate of cores planted with corn, which could result from movement along root channels. These findings are similar to those obtained by Madsen and Alexander (14), who found that roots did not affect movement of *Pseudomonas putida* or *Rhizobium japonicum* unless sufficient water was added to leach the cores. Van Elsas et al. (18) confirmed in a field study that survival and movement of *P. fluorescens* in soil was not influenced by roots in cropped and uncropped columns.

Most studies concerned with movement of bacteria in the soil profile have found that the bacteria remain in the upper layer of soil in the absence of saturated water flow. Van Elsas et al. (18) found the highest numbers in the upper layers, but numbers were detected at all depths to 25 cm. In nonsterile soil, Chao et al. (2) found that pseudomonads could not be detected in the rhizosphere more than 3 cm below the planted seed. Madsen and Alexander (14) also found that movement of *R. japonicum* or *P. putida* was not detected below 2.7 cm in absence of worm, plant, or percolating water. In this study we found the highest numbers consistently at the upper level of the cores. In addition, we could detect some bacteria at all depths after inoculation. Because we continually watered the cores to maintain field capacity, this could have contributed to the movement. The high numbers at lower depths obtained in cores sampled with minicores may have resulted from movement along the core plug. This suggests that if movement is to be studied in Versacores, the same cores should not be sampled with vertical mini-cores.

We did not find any competitive effect when the two recombinant strains were co-inoculated into the same soil samples. Therefore, the two organisms must not compete for the same sites or nutrients. Both species declined in number after inoculation in nonsterile soil, which could be caused by predation or competition from the indigenous soil population. Chao et al. (2) found that the biotic status of the soil markedly affected the colonization of microbes on roots. Bacteria competed more strongly in the rhizosphere for similar resources such as nutrients and space with other bacteria than with fungi. The known aggressive root-colonizing strain *Pseudomonas* sp. B8 did not establish rhizosphere populations as high as previously reported (6), possibly because of root zone temperature or the length of incubation. Fredrickson et al. (7) found that B8 achieved and maintained higher populations at lower temperatures as a result of increased competitive ability.

The more rapid population decline of both pseudomonads after the second inoculation in the preemptive colonization experiment was likely caused by desiccation of the soil surface at that time; the top of the cores dried out to only 5% moisture during this sampling period. This interpretation is consistent with the reported sensitivity of pseudomonads to soil drying (11,5).

While repacked cores are necessary for some experiments, this disruption of soil structure does change the microbial microenvironment, most notably by increasing the supply of nutrients and destroying natural channels. For studies of bacterial movement in which inoculation is at the top of the core, intact soil columns such as described by Van Elsas et al. (18) provide a better estimate of movement under field conditions.

In conclusion, Versacores were useful for tracking recombinant *Pseudomonas* strains and determining the effect of different environmental parameters on survival and dissemination. These factors are important for assessing the relative risk of releasing genetically engineered organisms into the environment.

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4.0 INTACT SOIL-CORE MICROCOSMS

In this section, intact soil-core microcosms were used to evaluate the fate, transport, and effects of genetically altered root-colonizing bacteria. This microcosm design has been used previously to evaluate the fate of inorganic utility wastes in terrestrial ecosystems and their effects on plant growth and ecosystem processes (1). A detailed protocol for obtaining soil-core microcosms is available as an ASTM standard guide (2).

The first section, Section 4.1, Intact Soil-Core Microcosms for Evaluating the Fate and Survival of Genetically Engineered Microorganisms in the Environment, was previously published in an abbreviated form in *Applied and Environmental Microbiology*, Vol. 55, pp. 198-202. The second section, Section 4.2, Fate of Root Growth-Inhibiting *Pseudomonas* sp. Tn5 Mutants in Intact Soil-Core Microcosms, has been submitted for publication.

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4.1 INTACT SOIL-CORE MICROCOSMS FOR EVALUATING THE FATE AND SURVIVAL OF GENETICALLY ENGINEERED MICROORGANISMS IN THE ENVIRONMENT*

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SUMMARY

Intact soil-core microcosms were studied to determine their applicability for evaluating the transport, survival, and potential ecosystem effects of genetically engineered microorganisms before they are released into the environment. Microcosms were planted with wheat and maize seeds and inoculated with *Azospirillum lipoferum* SpBr17 and SpRG20a Tn*5* mutants, respectively. Heat-killed cells served as controls. Above-ground plant biomass and leaf tissue nitrogen were measured to determine inoculation effects on plant growth. Microcosm leachate, rhizosphere soil, plant endorhizosphere, and xylem exudate were sampled for *A. lipoferum*

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Tn5 populations. Aphids and European corn borers were evaluated as potential vectors for *A. lipoferum* Tn5 dissemination. Whole pGS9 DNA probe hybridized to non-*Azospirillum* colonies in leachate, necessitating the use of a more specific DNA probe constructed from the *nptII* region of Tn5. *Azospirillum lipoferum* Tn5 mutant populations, determined by most-probable-number DNA hybridization, varied from undetectable to 10^5 per gram of dry root in the rhizosphere, with smaller populations detected in the endorhizosphere. Maize plants with established *A. lipoferum* Tn5 populations had higher nitrogen concentrations, but plant yield was unaffected. Rhizosphere populations of nitrifying bacteria (10^4 to 10^5/g dry soil) and *Rhizobium* (10^2 to 10^4/g dry soil) were unaffected by inoculation with *A. lipoferum* Tn5 mutants. These results indicate that microcosms maintain a high degree of ecosystem complexity and can serve as a representative model system to examine transmission through the ecosystem, rhizosphere and endorhizosphere colonization, potential displacement of other rhizosphere microorganisms, and effects on plant growth by genetically engineered microorganisms.

INTRODUCTION

Methods are needed to evaluate genetically engineered microorganisms (GEMs) in representative ecosystems to assess their fate and potential effects before they are released into the environment. Laboratory-contained microcosms that are composed of intact soil cores have potential as tools for risk assessment before field testing. Current philosophy in regard to the release of GEMs into the environment is that each organism should be regulated on the basis of the risk posed by that individual organism, rather than by how that organism was altered (27).

A standard test system must be adaptable to evaluate the release of a wide variety of microorganisms as well as detect possible detrimental effects on the ecosystem. Microcosms serve these purposes and can also be used to determine the efficacy of GEMs in a field-like situation without regulatory constraints. Intact soil-core microcosms have been used to study ecosystem stability (44), nutrient cycling (45), and the effect of utility wastes or other toxic substances on ecological processes (43,13,42). Microcosms have also facilitated the study of microorganism and pollutant interaction in aquatic (31) and sediment ecosystems (14). Chemical substances are dispersed, diluted, or degraded following their introduction into the ecosystem, but microorganisms can reproduce and increase in concentration under favorable conditions. Therefore, microorganisms have the potential to spread and colonize habitats that may be unique to each genotype.

Azospirillum lipoferum, a motile, diazotrophic root-colonizing bacterium that has been shown to increase plant biomass and nitrogen uptake when inoculated onto wheat (21) and maize (15,28), was chosen as the test bacteria for this study. *Azospirillum* spp. are widely distributed in soils and can form asymbiotic associations with plant roots (32). The effects of *Azospirillum* inoculation on root development and function, which are variable, have been recently reviewed (29). The strains used in this study colonize the rhizosphere, endorhizosphere, and xylem of maize (24) and wheat (22).

Azospirillum Tn*5* mutants were selected to enhance their detection and enumeration in the microcosms. The presence of Tn*5* in the bacterial genome confers kanamycin resistance (18), which can be used as a selective marker and also as a unique DNA sequence to positively identify mutants by DNA hybridization with a Tn*5*-specific probe (11). In addition, Tn*5* mutants have been shown to have a competitive advantage over their parent wild type, even in the absence of any apparent selective pressure (4).

Transposon mutants can serve effectively in studies to evaluate environmental fate and effects from the release of GEMs because they have been widely used as cloning vectors (23,3), for mutagenesis studies of bacterial symbiosis with plants (36), for plant pathogen interactions (7,9), and for determining bacterial survival in soil (40). Moreover, transposon mutants of *Pseudomonas syringae* have potential use as biocontrol agents against Dutch elm disease (33). This study was initiated to determine the applicability of intact soil-core microcosms for evaluating transport of microorganisms through the ecosystem, their colonization of the rhizosphere and endorhizosphere, displacement of other indigenous rhizosphere microorganisms, and effects on plant growth.

MATERIALS AND METHODS

Bacterial Cultures

Escherichia coli MV12 containing the suicide plasmid pGS9 (11) was used as the Tn*5* donor in matings. *Azospirillum lipoferum* strains SPRG20a [20a] and SpBr17 [17], ATCC 29708 and 29709, were purchased from the American Type Culture Collection, Rockville, Maryland.

Culture Media and Maintenance

Luria-Bertani (LB) broth (25), containing 25 ppm each kanamycin sulfate (Sigma Chemical Co., St. Louis, Missouri) and chloramphenicol (Sigma), was used for maintenance of *E. coli* MV12. Nitrogen-free Azospirillum medium was used as the basal medium (1). *Azospirillum* cultures were maintained on Azospirillum medium (AM), which consisted of nitrogen-free Azospirillum medium amended with 1.0 g NH_2Cl and 1.0 g yeast extract (Difco, Detroit, Michigan) per liter. *Azospirillum* Tn*5* mutants were routinely cultured on AM amended with 25 ppm kanamycin (Km) sulfate. Stock cultures of all bacteria were maintained at -80°C in 50% glycerol.

Bacterial Matings

Log-phase *A. lipoferum* cultures were centrifuged; cells were washed in 0.85% sterile saline and resuspended in sterile saline to obtain approximately 10^9 cells/ml. For matings, 0.1 ml *E. coli* MV12 donor and 0.4 ml *A. lipoferum* 20a or *A. lipoferum* 17 recipients were mixed, then transferred onto a 47-mm-diameter, 0.01-µm filter (Sartorious, Gottingen, F.R.G.) on 1.5% Luria-Bertani agar. After mating, cells were suspended in 0.5-ml sterile saline and spread onto 150 x 15 mm plates of AM containing 25 ppm Km to select for transconjugates.

Confirmation and Characterization of Tn*5* Insertion

Putative *Azospirillum* Tn*5* transconjugates were confirmed by spot-blotting an equal number of cells onto GeneScreen Plus (New England Nuclear Corp., Boston, Massachusetts) and hybridizing them with whole pGS9 ^{32}P probe. Plasmid DNA was labeled with ^{32}P-dCTP (New England Nuclear Corp.) using a nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Maryland). Plasmid gel electrophoresis was done by the procedure of Kado and Liu (19). Isolation of chromosomal DNA and restriction digests with *Eco*RI and *Hin*dIII (International Biotechnologies Incorporated, New Haven, Connecticut) were achieved as described by Sadowsky (35). Transfer of restricted DNA onto GeneScreen Plus was by vacuum blot (American Bionetics, Emeryville, California) by the manufacturer's protocol.

Microcosms and Inoculation

Intact soil cores for use as microcosms were obtained as described by Van Voris (42) using a steel coring apparatus containing polyethylene pipe (Phillips Petroleum Co., Dallas, Texas).

The intact soil cores, held inside the plastic pipe after removal from the driving tube, were placed in a housing on Buchner funnels [24-cm polypropylene (Dyna Lab, Rochester, New York) or 20-cm porcelain (Coors, Golden, Colorado)]. Two soil-plant combinations common to agricultural regions of eastern Washington were used. Cores (17.8 x 60 cm) of Palouse silt-loam soil (fine-silty, mixed, mesic Pachic Ultic Haploxeroll) from the Palouse region were planted with spring wheat (*Triticum aestivum*); cores (25 x 60 cm) of Burbank sandy loam soil (sandy, skeletal, mixed, xeric Torriorthent) common to the Columbia Basin were planted with maize (*Zea mays*). Common local seed varieties [NC+3390 maize (Wilbur-Ellis Seed Co., Pasco, Washington)] and Edwall spring wheat (USDA Research Station, Pullman, Washington)] were used.

Tn*5* mutant cultures were washed in 0.85% sterile saline and suspended in sterile deionized water to obtain a density of 9 x 10^8 and 4 x 10^8 CFU/ml for *A. lipoferum* 17 or 20a, respectively. Burbank soil microcosms were planted with four maize seeds per microcosm, each seed receiving 1.0 ml of an equal mixture of three *A. lipoferum* 17 Tn*5* mutants. Palouse microcosms were planted with five seeds per microcosm with each seed receiving 1.0 ml of a single *A. lipoferum* 20a Tn*5* mutant. Heat-killed suspensions of the same cells served as controls. There were six replicates for each treatment in a completely randomized design, and one-way analysis of variance tables were calculated to determine if statistically significant differences ($p \leq .05$) existed between measured parameters in inoculated versus control microcosms. Microcosms were maintained at a water potential of approximately -0.03 MPa and temperature of 22°C, as monitored by soil temperature and moisture probes on line to a datalogger (21x, Campbell Scientific Co., Logan, Utah). Photoperiod was set for 12 h with initial light intensity at the soil surface 400 microeinsteins/m^2 (4100 K Ultralume Lamps, Phillips Lighting Corp., Bloomfield, New Jersey).

Insects

Insects were introduced onto plants after the first sampling. Ten European corn borers (*Ostrinia nubialis*) (French Agricultural Research Service, Lamberton, Minnesota), at third to fourth instar, were placed on individual maize plants, and were confined to the lower stalk by loosely wrapping with chiffon. Approximately 50 to 100 aphids (*Schizaphis* sp.) indigenous to eastern Washington State were introduced onto wheat plants. After 12 and 21 days for corn borers and aphids, respectively, insects were sampled, macerated in sterile deionized water, and assayed for *A. lipoferum* Tn*5* mutants. Approximately 25 aphids were removed from the wheat leaf surface, and 4 to 9 corn borers were removed from the maize stem interior.

Sampling Frequency and Methods

Three replicate microcosms from each treatment were sacrificed at 4 and 9 weeks to determine bacterial concentration and movement. Soil cores were removed from the pipe intact, and root samples with adhering soil were taken randomly throughout the core with care to avoid sampling those growing at the interface between the core and the pipe. Standard methods were used for sampling rhizosphere soil (47). *Rhizobium leguminosarum* rhizosphere populations were enumerated by the most-probable-number (MPN) plant infectivity method of Weaver and Frederick (46) using peas (*Pisum sativum*) as the host plant. Nitrifying bacteria in the rhizosphere were enumerated by the MPN microtechnique of Rowe et al. (34). Endorhizosphere analysis for Tn*5* mutants (30) was accomplished by placing washed wheat and maize root sections in 1.0% chloramine T (Sigma) for 15 and 60 min, respectively, followed by a 30-min wash in sterile 0.1 *M*, pH 7.0 phosphate buffer. Roots were then rinsed three times with sterile deionized water, macerated, and evaluated for the presence of *A. lipoferum* Tn*5* mutants.

Xylem exudate was collected after cutting plants 5 cm from the base of the stem with sterile scissors and letting fluid collect on the stem surface for several minutes; the fluid was then removed with a sterile pipette tip and spread-plated immediately. Leachate was collected in sterile, acid-washed, deionized-water-rinsed, 2-liter Erlenmeyer flasks after saturating the cores with deionized water. Leachate was placed at 4°C within 12 h of breakthrough and entry into the flasks. Plant sampling for top biomass was conducted according to standard methods (17). Leaf nitrogen concentration was determined by Kjeldahl analysis (5) before maize tasseling and wheat booting.

Enumeration of *Azospirillum* Tn*5* Mutants

Rhizosphere, endorhizosphere, xylem exudate, insects, and leachate samples were analyzed for the presence and population of *Azospirillum* Tn*5* mutants. Colony blots (12) as well as MPN-DNA hybridization (11) were used for enumeration. All blots were then hybridized with ^{32}P-labeled probe and autoradiographed for enumeration. Because whole pGS9 probe proved nonspecific, a 30-base-pair sequence in the *nptII* region of Tn*5* was used as a probe. The *nptII* probe was synthesized (Model 380A, Applied Biosystems, Foster City, California) and purified by polyacrylamide gel electrophoresis with 15% polyacrylamide and

7 *M* urea according to the manufacturer's protocol. Kinase end-labeling (25) with [^{32}P]ATP (New England Nuclear) routinely achieved 10^8 dpm/µg DNA.

Soil Recovery

To determine the optimal medium for enumeration of *A. lipoferum* Tn*5* cells in soil, *Azospirillum* Tn*5* cultures were washed and spiked into Palouse soil at 10^3 and 10^5 cells/g soil. Soils were incubated at 4°C for 1 h. *A. lipoferum* Tn*5* cells were enumerated by plate count colony blot and MPN-DNA hybridization, as described previously, using nitrogen-free Azospirillum medium and AM amended with 150 and 200 ppm Km and cycloheximide (Sigma).

RESULTS

Azospirillum Tn*5* transconjugates were obtained at a frequency of approximately 10^{-9}. All Tn*5* mutants were stable after 50 generations of growth in AM broth without Km (data not shown). Both *A. lipoferum* 20a and 17 wild-type strains harbor two plasmids (Figure 1A). The *A. lipoferum* Tn*5* mutants chosen for this study harbored only the two indigenous plasmids, ensuring that pGS9 was not present as an extrachromosomal element (Figure 1A). Two of the mutants [20a-2 and 17-6], however, maintained Tn*5* in the smaller of the two indigenous plasmids (Figure 1B). These plasmids may be present in multiple copies and can be mechanically broken when subjected to the detection protocol used (19). Whole chromosomal DNA restriction digests (Figure 2) with *Eco*RI and *Hin*dIII, which give 0 and 2 cuts in Tn*5*, respectively (18), demonstrated that each mutant had a single copy of Tn*5* inserted uniquely in the genome (Figure 2). The one exception was mutant 17-6, which possessed pGS9 vector sequences, as demonstrated by the increase in the total number of *Hin*dIII-generated fragments hybridizing to pGS9, three of which correspond to the same size as *Hin*dIII-generated fragments of pGS9.

The optimal medium for the recovery of *Azospirillum* Tn*5* mutants when spiked into Palouse soil was AM containing 150 ppm Km and 200 ppm cycloheximide. Higher concentrations of antibiotics inhibited the Tn*5* mutants. When 150 ppm streptomycin was added in addition to 150 ppm Km, growth of the mutants was also inhibited; therefore, streptomycin was not used as an additional selective marker for the Tn*5* mutants. The use of

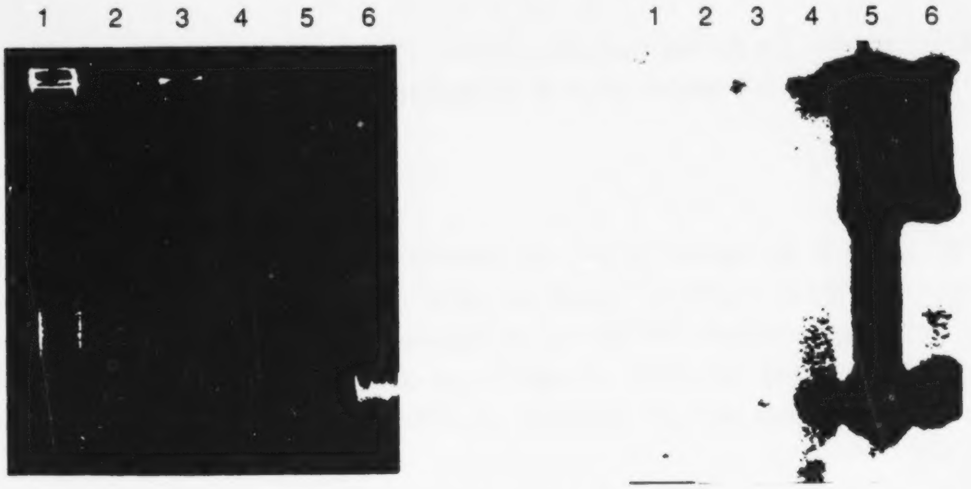

Figure 1. Plasmid gel electrophoresis (A) and autoradiogram of vacuum-transferred DNA hybridized with a pGS9 DNA probe (B). Lanes contain: *Pseudomonas putida* mt-2 with the TOL plasmid (117 Kb) as a size reference (1); *A. lipoferum* 17 wild type (2); and Tn*5* mutants 17-1 (3); 17-3 (4); 17-6 (5); and 20a-2 (6).

nitrogen-free medium selected against nonnitrogen fixers but also limited the growth of *Azospirillum* and therefore the amount of DNA available for hybridization when compared to AM, which provided nitrogen and yeast extract. All Tn*5* mutants used in this study were able to grow on nitrogen-free medium, indicating that nitrogen fixation functions were not affected by the transposon insertion. As many as 3.0% of the *Azospirillum* Tn*5* mutants spiked into soil were enumerated by colony blots; the limit of detection was approximately 10^3 CFU/g dry soil. Although not evident in the initial soil recovery experiment, leachate from microcosms receiving heat-killed cells contained bacteria other than *Azospirillum* that were homologous to pGS9. To determine if the homology resulted from Tn*5* sequences in indigenous soil bacteria or from vector sequences, a 30-base-pair sequence in the *nptII* region of Tn*5* was used as a probe. Isolated bacteria homologous to the probe when spot-blotted and hybridized with pCU101 (pGS9 without the Tn*5* DNA sequence) gave a strong hybridization signal, while a duplicate blot did not hybridize with the 30-mer *nptII* probe, indicating homology was with vector sequences (data not shown).

Rhizosphere populations of the *Azospirillum* Tn*5* mutants in the inoculated microcosms were variable, ranging from undetectable to 10^5/g dry weight (Table 1). Endorhizosphere populations as determined by MPN-DNA blots at midpoint sampling were below the detection limit for wheat roots, ranging from 5.5 x 10^3 to 6.5 x 10^3 CFU/g dry maize root in the Burbank soil microcosms. Populations determined by plate count and MPN manifold blot

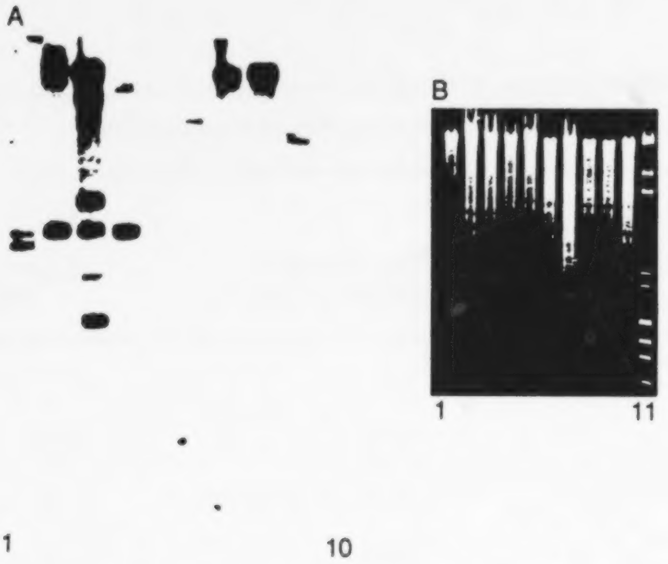

Figure 2. Agarose gel electrophoresis of chromosomal restriction digests of *A. lipoferum* Tn*5* mutants (A) and autoradiogram of vacuum-transferred DNA hybridized with a pGS9 DNA probe (B). Lanes 1 through 5 contain *Hin*dIII restriction digests of Tn*5* mutants 17-1 (1); 17-3 (2); 17-6 (3); 20a-2 (4); and *A. lipoferum* 17 wild type (5). Lanes 6 through 10 contain *Eco*RI restriction digests of Tn*5* mutants 17-1 (6); 17-3 (7); 17-6 (8); 20a-2 (9); and *A. lipoferum* 17 wild type (10).

were generally in agreement, although the latter gave more consistent and slightly higher (typically 50%) values. The background of bacteria with the ability to grow on AM amended with antibiotics ranged from nearly none (on insect samples) to 10^6/g soil in the Palouse rhizosphere; thus, the detection limit of mutant populations in rhizosphere samples was lower. No *Azospirillum* Tn*5* mutants were detected in leachate, xylem exudate, insects, or earthworms found fortuitously during microcosm sampling.

The mean plant leaf tissue nitrogen concentration (Table 1) was higher for the inoculated maize plants than for the controls. This difference was not statistically significant, although there was a relationship between the presence of *Azospirillum* and tissue nitrogen concentration in the replications (Table 1). Available nitrogen, as reflected in the nitrate concentration in the initial leachate from control microcosms, was significantly higher ($p \leq .05$) for Palouse soil (x = 287 ppm) than for the Burbank soil (x = 130 ppm). There was no significant difference in the number of tillers per wheat plant in the inoculated microcosms (1.32) versus control microcosms (1.29), or between plant top biomass of maize or wheat at either sample point (Tables 1 and 2). Although plant top biomass means of wheat in control and inoculated

Table 1. *Azospirillum lipoferum* Rhizosphere Populations, Plant Biomass, and Leaf Tissue Nitrogen Concentration, Before Plant Grain Fill

Treatment/ Replicates	*A. lipoferum* Rhizosphere (log MPN/g dry soil)	Plant Biomass Dry Weight (g)	Leaf Tissue N (%)
Maize/ Burbank soil			
1 - L[a]	4.55	6.78	3.02
2 - L	3.79	8.21	3.23
3 - L	bd[c]	12.42 (9.14 ±2.93)	2.64 (2.97 ±0.30)
1 - HK[b]	bd	8.06	3.21
2 - HK	bd	8.51	1.98
3 - HK	bd	14.11 (10.23 ±3.37)	1.96 (2.38 ±0.72)
Wheat/ Palouse soil			
1 - L	6.43	2.39	5.22
2 - L	bd	3.04	5.39
3 - L	bd	2.27 (2.57 ±0.41)	5.31 (5.30 ±0.09)
1 - HK	bd	2.65	5.17
2 - HK	bd	2.97	5.45
3 - HK	bd	2.86 (2.83 ±0.16)	5.00 (5.21 ±0.23)

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[a] Replicates receiving live *Azospirillum* Tn5 mutants.

[b] Replicates receiving heat-killed cultures.

[c] bd, below detection.

microcosms were not significantly different, a decrease in top biomass of maize plants with established rhizosphere *Azospirillum* populations was observed as each individual replication with measurable *Azospirillum* populations had lower plant biomass (Tables 1 and 2).

Table 2. *Azospirillum lipoferum* and *Rhizobium leguminosarum* Rhizosphere Populations and Plant Biomass After Plant Grain Fill

Treatment/ Replicates	*A. lipoferum* Rhizosphere Population (log MPN/g dry soil)	Plant Biomass Dry Weight (g)	Rhizobium Rhizosphere Population
Maize/ Burbank soil			
4 - L[a]	3.78	47.4	--
5 - L	bd[c]	64	--
6 - L	4.23	47.9 (53.1 ±9.4)	--
4 - HK[b]	bd	73.1	--
5 - HK	bd	57.4	--
6 - HK	bd	59.4 (63.3 ±8.6)	--
Wheat/ Palouse soil			
4 - L[a]	4.40	20.3	2.58
5 - L	3.75	18.5	3.23
6 - L	bd	13.4 (17.4 ±3.6)	2.42 (2.74 ±0.43)
4 - HK[b]	bd	15.6	3.31
5 - HK	bd	20.5	2.84
6 - HK	bd	14.7 (16.9 ±3.1)	4.30 (3.48 ±0.75)

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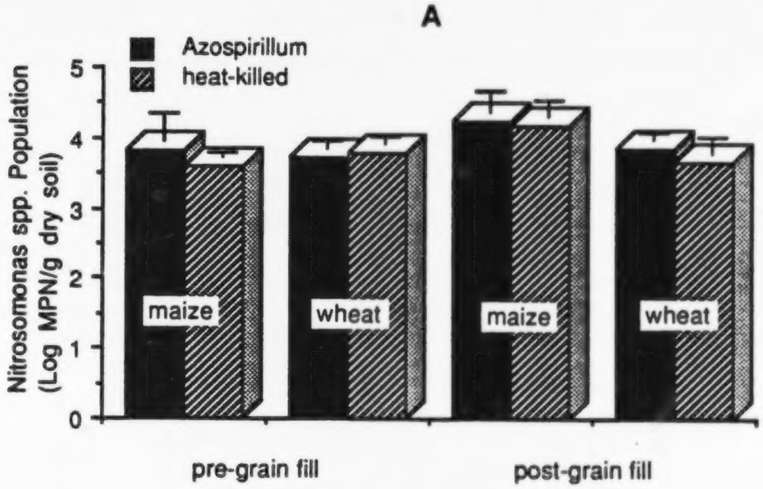
[a] Replicates receiving live *Azospirillum* Tn5 mutants.

[b] Replicates receiving heat-killed cultures.

[c] bd, below detection.

Rhizobium leguminosarum populations were present in the wheat rhizosphere of the Palouse soil microcosms, but were not influenced by inoculation with the *A. lipoferum* 17 Tn5 mutant (Table 2). Rhizosphere populations of *Nitrosomonas* spp. were similar for all treatments, while *Nitrobacter* spp. populations were consistently higher in the wheat rhizosphere than in the maize rhizosphere (Figure 3). Neither *Nitrosomonas* spp. nor *Nitrobacter* spp. rhizosphere populations were influenced by *A. lipoferum* inoculation in either the Palouse or Burbank soil microcosms (Figure 3).



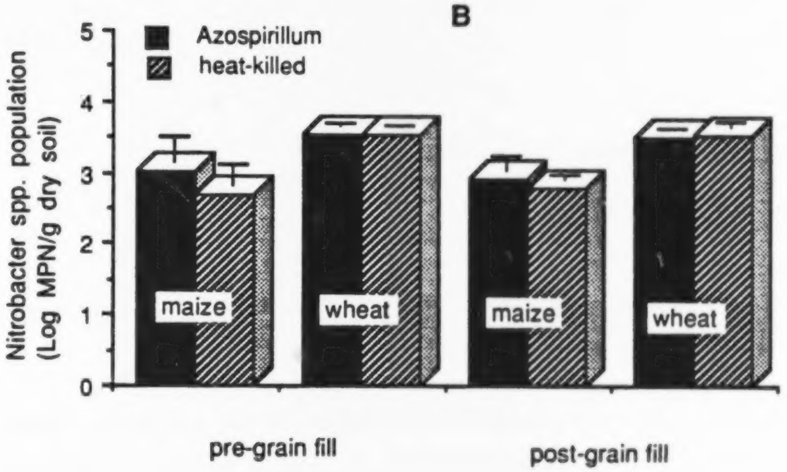


Figure 3. Rhizosphere soil populations of *Nitrosomonas* spp. (A) and *Nitrobacter* spp. (B) in microcosms inoculated with *A. lipoferum* Tn*5* mutants or receiving heat-killed cultures. Error bars represent 5% confidence intervals.

DISCUSSION

The frequency of Tn*5* insertions (10^{-9}) into the *A. lipoferum* strains, although low in comparison with other gram-negative bacteria (37), are similar to those previously observed for *Azospirillum* (41). Media and antibiotic concentrations used were not completely selective, allowing growth of *Azospirillum* but not suppressing growth of all indigenous soil microorganisms. This limited the detection of *A. lipoferum* Tn*5* mutants in soil to approximately 10^3/g soil.

Xylem exudate and insects had low background populations of kanamycin-resistant (Km^r) bacteria, as did Burbank microcosm leachate samples; hence, the Tn*5* mutants, if present and culturable, would have been readily detected by colony hybridization or MPN-DNA hybridization. Because of a high background of Km^r bacteria in Palouse soil able to grow on AM, the relatively slow growing *A. lipoferum* population may have been outcompeted, limiting its detection in the rhizosphere populations to approximately 10^3 CFU/g dry soil. Endorhizosphere samples and insect macerates from the maize plants contained a fungus that grew on AM agar with 200 ppm of cycloheximide. This fungal growth may have masked Tn*5* mutant populations in those samples.

MPN-DNA hybridization was more effective for enumerating *A. lipoferum* Tn*5* bacteria when high background levels of Km^r bacteria were present. In some samples, positive signals were obtained at low dilutions in microdilution plate blots while no positive colonies were detected on colony blots of dilution plates of the same samples. Presumably, the static broth provided *Azospirillum* more favorable conditions for growth and competition with other Km^r bacteria. *Azospirillum* seemed to be especially overgrown by the indigenous Km^r bacteria on solid media. Typically, the indigenous Km^r bacteria would appear after 2 days of incubation at 27°C where a minimum of 3 days was required for *Azospirillum* colonies. Additionally, not all colonies blotted efficiently, requiring their purification and isolation before hybridization with Tn*5* probe for confirmation.

The *nptII* probe was used because preliminary studies with whole pGS9 probe showed a high background of indigenous bacteria with homology to the suicide vector. Studies with *Pseudomonas putida* Tn*5* mutants added to dried and remoistened Palouse soil enumerated by MPN-DNA hybridization did not reveal homology of indigenous bacteria with whole pGS9 probe (11). In that study, either homologous bacteria were not able to grow on the media used for *P. putida* Tn*5* selection, or the bacteria were not present because of air-drying of soil.

Probe specificity and preliminary studies with appropriate controls are essential before usage for enumeration of specific bacterial populations in environmental samples.

Although *Azospirillum* rhizosphere populations were not uniform among replicate microcosms, a pattern was evident. Of the three replicate Palouse and Burbank microcosms receiving live inoculum at each sampling, two microcosms had detectable rhizosphere populations of *A. lipoferum* Tn*5*, while populations were below detection levels in one microcosm.

Variability of *Azospirillum* Tn*5* rhizosphere populations between replicates probably resulted from inherent differences in the microcosms. Intact soil cores, because they are removed as discrete pieces from the field, contain inherent variation that represents the complexity of that ecosystem. Examples of macroscopic differences observed in some microcosms and not others include visible layers of Mt. St. Helens ash from the 1980 eruption, the emergence of mushrooms, and the presence of earthworms. Microscopic differences could include variable distribution of indigenous soil bacteria (e.g., antibiosis) and distribution of nutrients.

The potential for niche displacement of rhizosphere populations of nitrifying bacteria and *Rhizobium* in the microcosms was also evaluated because of their involvement in biochemical nitrogen cycling and the ability of *Rhizobium* sp. to be stimulated in the rhizosphere of nonhost plants. These bacteria occupy the same physical medium (rhizosphere and bulk soil) so that any antibiosis or other deleterious effect on indigenous microbial populations from inoculation of *Azospirillum* Tn*5* mutants would likely be evident on those species. Although changes in the rhizosphere populations of nitrifying bacteria and *Rhizobium leguminosarum* were not observed in the inoculated microcosms, their consistent presence and low variability between replicates make them good indicators of ecosystem stability.

Plant growth effects from inoculation with *Azospirillum* have been attributed to N_2 fixation (26), production of plant growth hormones (39), and increased nutrient uptake at the roots (26). The expression of these traits and limited root colonization likely contribute to the variability in plant growth response common to *Azospirillum* studies. This suggests that the extent of *Azospirillum* root colonization may be the main factor controlling plant growth response.

Edaphic factors have been shown by others to strongly influence *Azospirillum* interaction with plants. *Azospirillum* inoculation of wheat in soils high in nitrogen or with high levels of nitrogen applied as a fertilizer have shown decreased effect on plant dry weight (20,21) and nitrogen incorporation into plant tissue (2) in comparison with those grown under low nitrogen conditions. Maize grown in Burbank soil, low in nitrogen relative to Palouse soil, showed a greater response to inoculation as indicated by an increase in plant leaf nitrogen in those replicates with detectable *Azospirillum* Tn*5* mutants (Table 1), although top dry weight was not affected. However, O'Hara et al. (28) tested seven nonindigenous strains of *A. brasilense* under greenhouse conditions and found that none extensively colonized the endorhizosphere and that yields were relatively constant, regardless of population size and location on roots.

Bacterial strain plant specificity must also be considered when examining the growth response of *Azospirillum*-inoculated plants. Soil and plant combinations used in this study may not have been conducive to expression of those bacterial genes that promote plant growth, such as N_2 fixation (8), or production of plant growth factors (39,16), by the specific *Azospirillum* strains used. As with wheat, plant genotype-*Azospirillum* interactions have been shown to be strain specific for maize (15).

Effects on plant growth (10) as well as on ecosystem processes (13) are likely to be dependent on the size of the microbial population. The inoculum concentration in this study was relatively small (10^8-10^9 CFU/seed) in comparison with most other studies. An optimum *Azospirillum* inoculation concentration has been shown for maize that was 10 fold less than the competing *Pseudomonas* population (10). Increasing the concentration of *Azospirillum* past the optimum population can offset beneficial effects to the plant.

In field plot experiments spanning 3 years, significant increases in wheat grain yield between treatments were not found even though there were increases as great as 31% in individual plots (2). This observed field variability is consistent with the variability in root colonization and plant growth effects observed in the microcosms, indicating that the intact cores have retained some degree of field complexity.

Undisturbed soil samples have been shown to allow some bacterial passage through the macropores (38), suggesting the potential for microbial leaching and subsequent transport in groundwater. The close association and adhering of *Azospirillum* with roots and low levels of inoculum likely limited their leaching through the soil cores in this study. *A. lipoferum* strains were chosen for their reported xylem mobility (24,22); hence, there was a potential for transmission from the plant vascular system to phytophagous insect vectors. In the specific *Azospirillum* soil-plant combination used in this study, extensive bacterial movement and colonization was not observed. Although the possibility exists that mutations incurred by the insertion of Tn*5* influenced the competitive ability of the bacteria, and therefore xylem mobility, it is more likely that the migration of *Azospirillum* mutants into the xylem is uncommon and occurs only under specific conditions. Transposon insertions have been shown to impair the relative competitiveness of *Rhizobium* (6) and, therefore, could have had a negative impact on the competitive ability of the *A. lipoferum* mutants used in this study. However, this is less likely for Palouse soil-core microcosms because a mixture of three different mutants was used for inoculation. Additionally, Tn*5* mutant bacteria have been provided a selective advantage in comparison with the wild type (4).

We have demonstrated that intact soil-core microcosms maintain many of the features useful for evaluating GEM transmission through the ecosystem, rhizosphere and endorhizosphere colonization, potential displacement of other rhizosphere microorganisms, and effects on plant growth. This is illustrated by the detection problems realized during hybridization of pGS9 to indigenous Km^r bacteria, whereas no such problem was encountered when using dried stock soil for preliminary recovery studies. This phenomenon is indicative of the higher order of ecosystem complexity maintained by use of intact soil-core microcosms (44). Other aspects of environmental release not evaluated in this study that may be evaluated using soil-core microcosms include GEM persistence and gene transfer. A major advantage of microcosms for GEM evaluation is their easy replication, necessary to determine the probability of finding a given effect or population for risk assessment. The use of intact soil cores allows the testing and comparison of GEM fate and effects in a number of different soil types, including those at the site intended for field testing.

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4.2 FATE OF ROOT GROWTH-INHIBITING *PSEUDOMONAS* SP. Tn*5* MUTANTS IN INTACT SOIL-CORE MICROCOSMS

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SUMMARY

Transposon Tn*5* mutants of a wheat root growth-inhibiting nonfluorescent *Pseudomonas* sp. were inoculated into intact soil cores to determine the fate and potential impacts from the release of this organism into agricultural ecosystems. Transposon mutants that no longer inhibited the growth of wheat roots or *Escherichia coli* through the production of a toxin (tox$^-$) as well as Tn*5* mutants similar to the wild type (tox$^+$) were inoculated into the surface soil of intact soil-core microcosms; the soil was spring wheat planted, and Tn*5* mutant populations were monitored over time in the bulk soil, rhizosphere, and rhizoplane with depth. Populations were also monitored in core leachate and in the gut of earthworms. The population of the introduced Tn*5* mutants declined over time in the surface soil but was present in significant numbers in the wheat rhizosphere and rhizoplane throughout the 60-cm soil-core depth. Rhizoplane populations of the tox$^+$ Tn*5* mutants were significantly higher than populations of tox⁻ mutants at the seedling stage but were not significantly different at the flowering stage. The Tn*5* mutants were transported through the core with percolating water and were present in the gut of earthworms. Plant growth as well as indigenous rhizoplane populations of *Rhizobium leguminosarum*, associative N_2-fixing bacteria, and nitrifying bacteria were not affected by inoculation with the Tn*5* mutants. These experiments indicate that intact soil cores may have utility for assessing the fate and potential ecosystem impacts from the release of genetically engineered microorganisms to the environment.

INTRODUCTION

Plant-associated, and in particular rhizosphere-associated, bacteria have great potential for biotechnological applications in agriculture for biological control of plant diseases and for improving plant nutrition and providing plant growth factors (10). Microorganisms used in these applications may be improved through genetic engineering; however, concern about the release of genetically engineered microorganisms (GEMs) to the environment requires that the environmental risks be evaluated before a large-scale release. Microcosms have been identified as potential tools for both research and risk assessment concerning the release of GEMs to the environment (7). Microcosms have previously been used to assess the fate and impacts of released chemicals to the environment (40,19) and the interactions between pollutants and microorganisms (30). The intact soil-core microcosm was developed to evaluate the fate and ecological effects of chemicals in the environment (39) and, more recently, modified to evaluate the fate and environmental impacts from the release of GEMs (3). This research investigates the use of intact soil-core microcosms for evaluating the fate and potential environmental impacts from a genetically altered root-colonizing pseudomonad.

The use of no-till cultivation for wheat, and cool, wet weather in the spring months, favors the growth of nonfluorescent *Pseudomonas* sp. that inhibit wheat root growth (13). These bacteria can heavily colonize the rhizoplane of winter wheat and stunt root growth (15,16) and can achieve high population densities on crop residues (17,36). These wheat root growth-inhibiting *Pseudomonas* sp. have been shown to produce a low molecular weight toxin that inhibits the growth of wheat plants as well as some bacteria and fungi (4). This toxin appears to be a secondary metabolite, because activity can be detected in cultures grown under a wide range of conditions (5). These bacteria extensively colonize peas, oats, and lentils, but are not deleterious to these plants (17). Mutants that no longer produce the toxin (15) may have potential as a biocontrol agent to displace the wild type, as has been accomplished for ice nucleation-minus *Pseudomonas syringae* for control of frost damage (24). Also, other root-colonizing bacteria have been isolated that specifically inhibit the growth of weeds (8). Because these bacteria can exhibit a deleterious effect to a narrow host range and are aggressive root colonizers, they may also have potential for biotechnological applications in agriculture.

The specific objectives of this study were to measure the colonization of *Pseudomonas* sp. Tn*5* tox$^+$ and tox$^-$ phenotypes in the wheat rhizoplane, rhizosphere, and bulk soil with depth; determine the potential for transport of the introduced bacteria through the intact soil cores with percolating water and root growth; determine the potential for antagonism of several physiological groups of rhizosphere bacteria; and to determine if earthworms can serve as vectors of the introduced bacteria in soil.

MATERIALS AND METHODS

Bacterial Strains and Media

Toxin-producing (tox$^+$) and toxin-deficient (tox$^-$) Tn*5* mutants of wheat root growth-inhibiting *Pseudomonas* sp. isolate RC1 were obtained from Dr. Lloyd F. Elliott [U.S. Department of Agriculture, Agriculture Research Service (USDA-ARS), Pullman, Washington]. The tox$^-$ phenotype indicates the mutant is unable to inhibit root and *E. coli* growth. Described elsewhere are the procedures and vectors used for generation of transposon mutants (6), screening for toxin production (5), mutant growth characteristics (4), and chromosomal location of Tn*5* insertion in the bacterial genome (45). Three mutants of each phenotype (tox$^+$ or tox$^-$) with unique Tn*5* insertions were selected for inoculation of soil cores to increase the probability of introducing an organism that was not severely debilitated in its competitive ability (3). The medium of Sands and Rovira (31) amended with 500 µg/ml of kanamycin (Sands Km^{500}) was used for enumeration of mutants in microcosm samples and for routine culturing. Tryptic soy agar (TSA) (27) was used to enumerate total bacterial numbers.

Microcosms and Inoculation

Intact cores (17.5 x 60 cm) of Palouse soil (fine-silty, mixed mesic Pachic Ultic Haploxerolls) were obtained for use as microcosms and maintained as previously described (3). Briefly, intact soil cores were collected using a steel coring apparatus containing polyethylene pipe (Phillips Petroleum Co., Dallas, Texas). The intact soil cores, held inside the plastic pipe after removal from the driving tube, were placed in a housing on 20-cm-diameter Buchner funnels that were connected to sterile, acid-washed, deionized water-rinsed, 2-liter Erlenmeyer flasks for leachate collection. Six replicates of each treatment, including tox$^+$, tox$^-$, and uninoculated control, were arranged in a completely randomized design. The top 15 cm of soil was removed from each of six replicate microcosms and combined. The soil was mixed for 15 min in a rotating soil mixer then placed at 4°C until inoculated. Six 150 x 25 mm Sands Km^{500} agar plate cultures of each mutant were harvested separately, 48 h after inoculation, by adding 10 ml of sterile deionized water and dislodging the cells with a glass rod. The bacterial suspensions were transferred to a sterile bottle, diluted with sterile deionized water, mixed, and placed on ice. Optical density (600 nm) of each inoculum was measured and adjusted to obtain the same relative cell concentrations for each culture. Inoculated treatments consisted of a mixture of three tox$^+$ mutants (A4-6, B4-7, I3-4) or a mixture of three tox$^-$ mutants (RC-D, RC-E, RC-F). The three different mutants for each phenotype (tox$^+$ or tox$^-$) were mixed in equal portions and diluted to 200 ml final volume before soil inoculation. The control treatment received 200 ml of sterile deionized water.

Soils were inoculated and mixed in a rotating mixer for 30 min, stopping twice to break up the soil aggregates, to ensure a thorough mixing of soil and inoculum. Equal amounts (approximately 2.3 kg) of inoculated soil were returned to the replicate microcosms. Six seeds of spring wheat (*Triticum aestivum* L. Edwall) were planted in each microcosm and thinned to four plants after germination. Microcosms were watered with sterile deionized H_2O to maintain a water potential of approximately -0.03 MPa with a growth temperature of 22°C, as monitored by soil temperature and moisture probes on line to a datalogger (21x, Campbell Scientific Co., Logan, Utah). Photoperiod was set for 12 h with an initial light intensity at the soil surface of 400 microeinsteins/cm (4100 K Ultralume Lamps, Phillips Lighting Corp., Bloomfield, New Jersey). Two earthworms (*Lumbricus* sp.) were introduced into microcosms after 3 weeks of wheat growth to assess their potential as vectors of the introduced bacteria.

Nondestructive Microcosm Sampling

Pseudomonas sp. Tn*5* mutant populations in the surface soil were measured over time. Approximately 15 g of soil was removed from the top 15 cm of the microcosms at each sampling point, taking care not to remove or disturb plant roots; 10 g was used for bacterial enumerations and 5 g for moisture content analysis. Two wheat seedlings with intact roots were removed on day 21 to determine the extent of root colonization by the Tn*5* mutants. Methods used for rhizosphere and rhizoplane sampling are described in detail elsewhere (15).

Briefly, for rhizosphere soil samples, the soil adhering to roots was washed off the root into a sterile dilution blank. The washed roots were then macerated with a mortar and pestle for the rhizoplane samples. Initial dilutions were shaken for 20 min on a gyratory shaker at 250 rpm. Rhizosphere populations of native *Rhizobium leguminosarum* were determined by the most-probable-number infectivity method using *Pisum sativum* as the host plant (41).

Twenty-five days after inoculation, microcosms were leached with sterile deionized water (250 ml) applied twice daily (9 to 12 h apart) until leachate was observed in 2-liter Erlenmyer leachate collection flasks. Leachate was decanted into a sterile container and placed at 4°C within 12 h of soil breakthrough and entry into the flasks. Leachate samples were serially diluted and plated onto Sands Km^{500} to enumerate *Pseudomonas* Tn*5* mutant populations.

Destructive Microcosm Sampling

Microcosms were sacrificed on day 54, or the boot stage of wheat. Wheat plants were cut at the soil surface and above-ground plant biomass determined. Two cuts were made along the length of the polyethylene pipe enclosing the intact soil cores; the pipe was removed, and the intact core was separated into five sections according to depth (0-15, 15-25, 25-35, 35-45, and 45-55 cm). The outer 1 cm of soil, which was next to the pipe, was pared to remove soil and roots at the pipe interface. Roots with adhering soil were removed from each section and processed as previously described for day 21, except rhizoplane sample roots were macerated for 90 sec in a tissue homogenizer (Sorvall Omni-Mixer, E. I. Du Pont De Nemours and Co., Newtown, Connecticut). At each depth the soil remaining in the soil core after rhizoplane and rhizosphere sample collection was mixed and retained as a bulk soil sample.

Pseudomonas sp. Tn*5* mutant populations in the rhizoplane, rhizosphere, and bulk soil were determined at each depth by dilution plating on Sands Km^{500} medium. Total bacterial numbers in the rhizoplane were determined by dilution plating on TSA. Rhizoplane populations of N_2-fixing bacteria in the soil-core surface layer (0 to 15 cm) were enumerated by the MPN-acetylene reduction method (22), and *R. leguminosarum* was enumerated as described earlier (day 21). Ammonium-oxidizing bacteria on the rhizoplane were determined by the MPN method of Schmidt and Belser (32).

Earthworms were removed from the soil cores during sampling and placed in autoclaved (40 min, 2 consecutive days) Palouse soil containing 1% ground wheat straw (a water potential of -0.03 MPa) and incubated at 4°C to enrich (17) for the introduced bacteria. *Pseudomonas* sp.

Tn5 mutant populations were measured in this soil on Sands Km^{500} after a 7-day incubation. Earthworms were removed after 23 days in the wheat straw-amended soil, washed, surface disinfected by placing in 1% Chloramine T (Sigma Chemical Co., St. Louis, Missouri) for 1 min., and dissected to sample the gut for the presence of the introduced bacteria.

Data were analyzed as a completely randomized design with six replicates. A protected least significant difference (LSD) was conducted at the $p \leq .05$ level of significance for all treatment mean comparisons (35).

RESULTS

The insertion site of *Tn5* into the genome was unique for each of the three tox^+ or tox^- mutants selected for each treatment (data not shown), thereby increasing the probability of including mutants that were not severely impaired in competitive ability. Sands Km^{500} provided excellent selection for the *Tn5* mutant bacteria because no growth occurred on plates from dilutions of the control microcosms. This level of kanamycin was chosen because it was the highest level that could be used without reducing the plating efficiency of the transposon mutants (data not shown). The stability of *Tn5* in these bacteria was not investigated in this study; however, previous studies have shown that *Tn5* was relatively stable with regards to excision and loss from the chromosome of *P. putida* and *R. leguminosarum* cv. *phaseoli* grown in soil (18).

Bulk surface soil (0 to 15 cm depth) populations of the *Pseudomonas* *Tn5* mutants declined slowly from approximately 1 x 10^8 CFU per dry gram of soil at the time of inoculation to 4 x 10^5 CFU/dry g after 30 days and 3 x 10^4 CFU/dry g after 54 days (Figure 1). Populations of tox^+ and tox^- mutants declined in the surface bulk soil at an identical rate. The LSD of the mean bulk soil populations of the *Tn5* mutants in the inoculated soil layer at the day 54 sampling (Figure 2) were considerably larger than the same value in Figure 1. This difference in the LSD value occurred because all depths were statistically analyzed together for Figure 2. A marked increase in variability occurred below the inoculated 0- to 15-cm layer; some bulk soil samples in the lower sections of the soil core had populations less than the detection level of 200 CFU/dry g soil.

At the day 21 sampling, the wheat seedling rhizoplane population of *Pseudomonas* *Tn5* mutants was more than 10 fold higher than in the rhizosphere, which was 10 fold higher than in

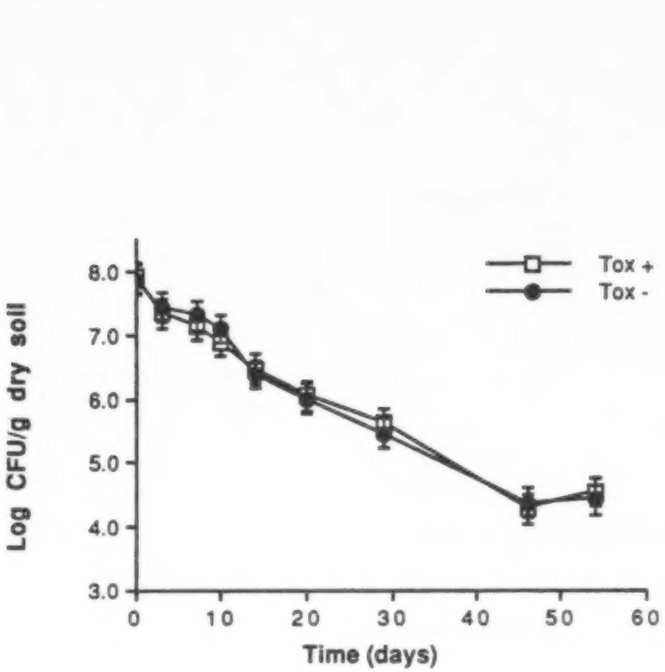

Figure 1. Populations of *Pseudomonas* sp. Tn*5* tox$^+$ and tox$^-$ mutants in the inoculated surface soil of soil-core microcosms over time. Error bars represent significant differences ($p \leq .05$).

the bulk surface soil (Figure 3). The mean tox$^+$ populations in the bulk or rhizosphere soil were not significantly different from tox$^-$ populations (Figure 3). However, mean rhizoplane populations of tox$^+$ bacteria were significantly higher than tox$^-$ populations. Leachate appeared in the collection flasks after approximately 5 days of leaching. Between 2.5 and 3.5 liters of sterile water was used to leach each microcosm, and the volume collected was consistently between 90 and 150 ml. Eleven of 12 inoculated microcosms had detectable populations of Tn*5* mutants in their leachate, which averaged more than 10^3 CFU/ml (Figure 3).

Distribution of *Pseudomonas* Tn*5* mutants by depth in the rhizoplane, rhizosphere, and in bulk soils at day 54 is shown in Figure 2. Differences in rhizoplane populations of tox$^+$ or tox$^-$ Tn*5* mutants were less than 1.5 log units over the length of the soil core. The rhizosphere populations of the Tn*5* mutants at each depth paralleled those of the rhizoplane but were approximately 10 fold lower. Bulk soil populations declined sharply from approximately 3 x 10^4 CFU/g in the inoculated surface soil to approximately 10^2 CFU/g in the four deeper sections. There was no significant difference between Tn*5* mutant tox$^+$ and tox$^-$ rhizoplane, rhizosphere, or bulk soil populations with depth except in the rhizosphere at the 15- to 25-cm depth. At microcosm harvest (day 54), there was no significant difference in plant biomass between treatments. Average shoot biomass was 7.83 g (dry weight) per microcosm for control, 8.41 for tox$^-$, and 9.08 for tox$^-$ treatments with an LSD value ($p \leq .05$) of 1.89 g.

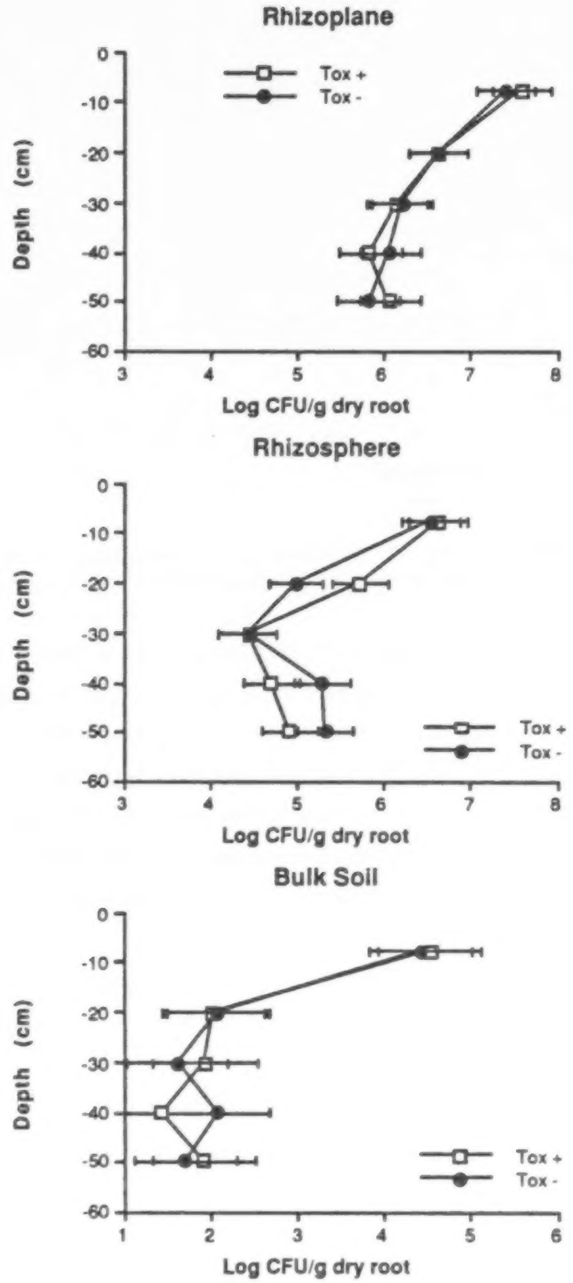

Figure 2. Rhizoplane, rhizosphere, and bulk soil populations of *Pseudomonas* sp. Tn*5* tox^+ and tox^- mutants with depth in the intact soil cores at day 54. Error bars represent significant differences ($p \leq .05$).

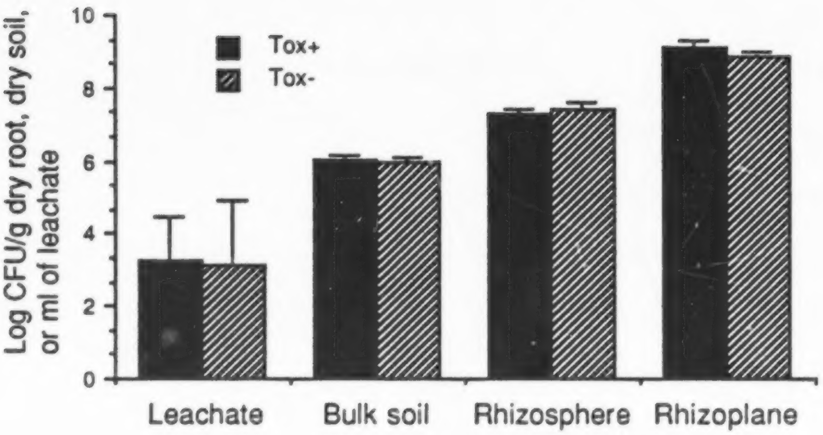

Figure 3. Populations of *Pseudomonas* sp. Tn*5* tox$^+$ and tox$^-$ mutants in microcosm leachate, bulk, and rhizosphere soil and in the rhizoplane at the wheat seedling stage (day 21). Error bars represent significant differences ($p \leq .05$).

Because all wheat roots were not recovered, a total root mass could not be calculated. However, bacterial inoculation caused no obvious stunting of root growth.

Total bacterial populations (on TSA) in the rhizoplane tended to follow a pattern similar to that observed for the transposon mutants (Figure 4). Populations declined 10 fold or less on roots from the surface to those sampled from the lower sections. The total population tended to be lower in the tox$^-$ treatments at all depths, but significant differences were found only at the 15- to 25-cm depth. Extreme variability was found when the tox$^+$ and tox$^-$ populations on the rhizoplane were represented as a percent of the total bacterial population as determined on TSA. Values ranged from 8% to virtually 100% for both, demonstrating that both inoculated bacteria constituted a significant portion of the total bacterial population on the rhizoplane.

Inoculation with the different inhibitory pseudomonad Tn*5* mutants had no significant ($p \leq .05$) effect on rhizoplane population sizes of *R. leguminosarum*, N_2-fixing bacteria, or ammonium-oxidizing bacteria in the surface (0 to 15 cm) soil. Rhizoplane populations of *Rhizobium leguminosarum* biovar *viceae* ranged between 7.23 ± 0.43 and 7.43 ± 0.35 log MPN/g dry root, free-living N_2-fixing bacteria ranged between 4.82 ± 0.29 and 5.05 ± 0.51 log MPN/g dry root, and populations of ammonium-oxidizing bacteria ranged from 4.25 ± 0.24 to 4.66 ± 0.20 log MPN/g dry root. In addition, *R. leguminosarum* populations in the

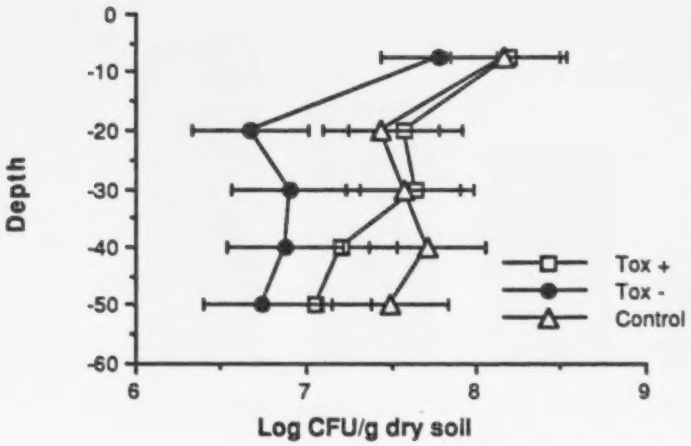

Figure 4. Rhizoplane populations of total bacteria (on TSA) with depth at day 54 in soil-core microcosms inoculated with Tn*5* mutants and uninoculated controls. Error bars represent significant differences ($p \leq .05$).

rhizosphere of tox$^+$ treatments (7.17 log MPN/g dry soil) were not significantly different (LSD = 0.52; $p \leq .05$) from populations in the tox$^-$ treaments (7.37) or in the uninoculated microcosms (6.91) at the day 21 sampling.

In the autoclaved and wheat straw-amended soil, populations of tox$^+$ and tox$^-$ Tn*5* mutant bacteria were 10^5/g dry soil or greater 7 days after earthworms from microcosms were added. Three of six earthworms sampled after 23 days had *Pseudomonas* Tn*5* mutants present in their gut. No growth was observed on Sands Km^{500} receiving dilutions from earthworms sampled from uninoculated microcosms or their straw-amended soil.

DISCUSSION

Inoculation of plow layer soil with the three genotypically distinct Tn*5* mutants of tox$^+$ or tox$^-$ root inhibitory pseudomonads resulted in colonization of the wheat rhizosphere and rhizoplane of roots sampled from all depths. The use of Sands Km^{500} provided excellent selection for the *Pseudomonas* Tn*5* mutants, thus eliminating background growth even at the lowest dilutions. There was no difference between tox$^+$ and tox$^-$ mutant populations in the bulk soil at any sampling time. Populations of tox$^+$ mutants colonizing the wheat rhizoplane were significantly higher than populations of tox$^-$ mutants 21 days after seeding and inoculation. Rhizosphere populations of tox$^+$ and tox$^-$ mutants were not statistically different at either sampling. The rhizoplane represents the region of the root zone where the density of inhibitory pseudomonads is highest (15) and the concentration of the antibiotic produced by these bacteria would probably be greatest. Thus, the greatest potential for antagonism of the indigenous microflora would be in the rhizoplane.

The root-colonizing pseudomonads introduced at high population densities in the surface soil of the microcosms declined more than 1000 fold over a 54-day period (see Figure 1). These results agree with the decline of *Pseudomonas* sp. in bulk soil after the introduction of large populations of laboratory-cultured bacteria into uncropped soil in other studies (38,28). In the later stages of plant growth, that is, the boot stage, the rhizoplane as well as the rhizosphere population densities of the Tn*5* mutants in the surface layer had declined by 10 fold or greater (see Figures 2 and 3). The rhizosphere bacterial population of wheat has been shown to decrease with the age of the plant and to decline sharply during flowering (33). A rifampicin- and nalidixic acid-resistant fluorescent pseudomonad mutant introduced as a seed inoculum on wheat declined from approximately 10^8 to less than 10^4 CFU/g root from germination to harvest (42). However, it cannot always be predicted a priori that bacteria introduced into soil will rapidly die off (12,9), indicating that bacterial decline in soil is dependent on the type of bacteria as well as environmental conditions.

Results of this study indicate that inoculation of soil with either tox$^+$ or tox$^-$ wheat root growth-inhibiting *Pseudomonas* sp. did not affect *R. leguminosarum* rhizosphere populations at the seedling stage or rhizoplane populations of *R. leguminosarum*, N_2-fixing bacteria, or ammonium-oxidizing bacteria at boot stage. The *Pseudomonas* sp.-produced toxin has been shown not to affect the growth of *Rhizobium* sp. in vitro (unpublished data).

Toxins have long been assumed to provide a selective advantage to microorganisms that produce them. While there have been many demonstrations of antibiosis in vitro, experimental evidence for the selective advantage of toxin-producing bacteria in natural systems is, with a few exceptions (1,23), limited. The use of intact soil cores as microcosms provides a means to evaluate such interactions in the laboratory while retaining complexity analogous to the field. Evaluation of intact soil cores as microcosms to study the effect of fly ash on plant uptake of trace elements demonstrated that soil-core microcosms could serve as reasonable analogs of the field (37,40).

The Tn*5* mutant bacteria introduced into the surface soil layer moved through the soil core with percolating water, as evidenced by the presence of these bacteria in the leachate (see Figure 3). Because the cores were gradually saturated by the application of small quantities of water over a long period of time, it is unlikely that significant volumes of water channeled along the soil-pipe interface and into leachate collection flask. If channeling had occurred, water would likely have been observed after the first few volumes (250 ml) of water had been added rather than after the fourth day when 2.5 to 3.5 liters of water had been added to the various microcosms. This evidence, plus the tight fit of the soil core in the polyethylene pipe and the fact that the soil expands as it absorbs water, indicate bacterial transport through the soil core. In addition, distribution with depth of *Pseudomonas* Tn*5* mutants in bulk soil (see Figure 3) indicates bacterial movement occurs through the core itself. At sampling, soil and roots at the soil-polyethylene pipe interface were pared to eliminate any influence from bacteria transported by channeled water. Smith et al. (34) concluded that *E. coli* and Cl^- moved through the center of intact cores faster than at core edges and that transport potential through intact soil cores is greater than through soil that has been mixed and packed.

Experiments using sieved, packed soil columns demonstrate that pseudomonads are more susceptible to water-mediated transport than other bacteria. Madsen and Alexander (25) found no vertical dispersal of bacteria occurred below the inoculated layer without some transporting agent (i.e., water) and that *P. putida* was transported farther and in greater numbers than *R. japonicum*. Other studies have demonstrated *Pseudomonas* spp. to be more prone to movement through soil than other bacterial species such as *Bacillus* and *Azotobacter* (44). However, even the movement of *P. fluorescens* through sterile soil was restricted in all but very wet conditions (44).

Populations of nonfluorescent Tn*5* mutants found in leachates of the intact soil-core microcosms are comparable to those observed by Martin (26) in a study evaluating the leaching of bacteria from wheat roots. That study also found fluorescent pseudomonads to be partitioned into leachate to a greater degree than other rhizosphere bacteria. Earlier work with intact soil-core microcosms of the same soil type used in this study as well as with a more coarsely textured soil showed that *Azospirillum lipoferum* Tn*5* mutants introduced as a seed inoculum on wheat or corn roots were not detected in leachate. However, the size and method of the inoculum and the extent of root colonization was considerably less in that study (3).

The pseudomonad Tn*5* mutants colonized roots in the lower section of the soil core (55 cm); however, the mode of transport to roots in this zone is unknown. Because the soil cores were leached at day 25 and the bacteria were present in the bulk soil of the lower core sections at day 54, bacterial colonization of roots in these zones may have resulted from percolating water. In a field study with potato plants, irrigation increased rhizosphere populations of *P. fluorescens* 10 fold whereas no increase was found in nonrhizosphere soil (2). The decline in populations of the introduced bacteria on wheat roots with depth is typical of that observed in other studies and is not indicative of the mode of movement of bacteria to a particular root segment. Bacteria introduced as seed inoculants on wheat were present in progressively lower numbers with increasing depth from the seed in the absence of vertical water movement in a laboratory system designed to control soil matric potential (21). A similar decline with depth of inoculated root-colonizing fluorescent pseudomonads has been observed in the field (43).

Deleterious effects on plant growth were not observed in this study because population densities were considerably less than the 10^6 CFU/mg dry root necessary for growth inhibition (15). In addition, other factors that contribute to optimal root colonization and growth inhibition, such as low temperature (14), susceptible wheat cultivar (13), or the presence of crop residues (18), were not provided.

The presence of the *Pseudomonas* Tn*5* mutants in the digestive tract of earthworms present in the soil cores suggests that earthworms may act as vectors in disseminating bacteria introduced into soils. Also, the population of introduced bacteria could be significantly increased upon passage through the earthworm; Parle (29) found that bacterial and actinomycete numbers increased by more than 100 fold over numbers found in the soil during passage of food through the worm gut. Earthworms have been shown to increase the transport of bacteria in soil as well (25).

The transposon Tn*5* has been shown in this study and others (38) to provide an excellent phenotypic tag for tracking the fate of microorganisms in the environment. Tn*5* could provide a genotypic tag as well, and may be used for direct (20) and indirect (18) analysis of populations in soils using DNA-hybridization techniques. Further, transposons can create clean mutations that may be of use in studying the effect of specific mutations on environmental competency or pathogenicity of a microorganism (23,11,45). Such transposon mutants could also have potential for commercial applications in agricultural biotechnology (23).

This study has demonstrated that toxin production can influence the rhizoplane population of a root-colonizing *Pseudomonas* sp. The fact that no differences in rhizoplane, rhizosphere, or bulk soil populations of tox$^+$ or tox$^-$ root growth-inhibiting *Pseudomonas* sp. Tn*5* mutants were observed except in the rhizoplane of wheat seedlings suggests that the tox$^-$ phenotype may be used as a biocontrol agent to suppress such deleterious bacteria in the rhizoplane.

Intact soil cores have been used in this study as analogs of the field to study microbial fate and effects of root-associated bacteria on indigenous bacterial populations in an agricultural ecosystem. The dissemination, survival, and impacts of an introduced genetically altered microorganism should be of value in future research involving genetically engineered microorganisms in intact soil-core microcosms intended for release to the environment. Calibration of microcosm with the field environment would improve the utility of microcosms for predicting behavior in the field (30), and, thus, risk assessment.

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5.0 DNA PROBE METHODS

Sensitive methods of detection for GEMs and their rDNA are necessary to adequately evaluate the risks associated with their release to the environment. The ability to distinguish between "die-off" or complete microbial death (and DNA degradation) and "dieback" or population decline below detectable levels (and DNA survival) (13) is critical to these studies. Traditional methods used to detect and enumerate microorganisms in the environment include techniques of direct plating on selective or differentiating media, direct microscopy, serology, immunofluorescence, immunoradiography, enrichment cultures, and analysis of characteristic cellular components (e. g., fatty acids, proteins, rRNAs). The advantages and disadvantages of these methods have been briefly reviewed by McCormick (13).

DNA probes are highly specific (6) and have been used to detect *Salmonella* spp. (4) and *Yersinia* spp. (7) in food; catabolic genes in sediments (17); *Bradyrhizobium japonicum* with either a naturally occurring DNA sequence or a region of Tn*5* (8), and *Pseudomonas cepacia* with a repeated region of DNA used as the probe (18). An additional advantage to using DNA probes to study the fate of GEMs is that some techniques that employ them are independent of culturing (8,18). A recent review provides an overview of methods for detecting recombinant DNA in the environment (11), and the applications, advantages, and disadvantages of DNA probes for the study of microbial ecology were discussed by Holben and Tiedje (9).

Detection of gene transfer in the environment presents several experimental obstacles. The analysis of gene transfer in the environment typically requires the addition of selectively marked donor and recipient strains that can used with selective media to detect donors, recipients, and transconjugants. However, only a small proportion of the bacterial population in the environment, and of soil in particular, is culturable (3). In addition, microorganisms may exist in a viable but not culturable state (2,10), so the recombinant DNA sequences that they carry cannot be detected by traditional methods. This presents the problem that gene transfer that could occur between introduced microorganisms and the indigenous microbial population might not be detected by standard cultural methods. Only recently have methods been developed that are independent of culturability. Holben et al. (8) isolated DNA directly from bacteria removed from the soil and detected specific genetic sequences of bacteria present at densities as low as 4 x 10^4 cells per gram of dry soil. By using restriction endonucleases in conjunction with Southern hybridization analysis, gene transfer and genetic recombination with indigenous microbial populations could be detected. The detection limit (4 x 10^4 cells/g dry soil) of this method, unfortunately, is relatively high, which limits its use for detecting gene transfer in microcosms and the field. However, recent advances in gene amplification using the *Taq* polymerase chain reaction method (16) can increase the sensitivity of direct detection of specific gene sequences in environmental samples by a factor of 10^4 or greater (18). Microcosms, as a representative portion of the ecosystem, could be used to evaluate the sensitivity and specificity of DNA probe methods to directly detect specific DNA sequences in terrestrial environments and to assess the transfer of rDNA to indigenous species under expected and extreme environmental conditions.

This section describes two recent advances in the application of genetic probes for detecting genetically engineered bacteria soil. In Section 5.1 is described the work of Jansson et al. in which direct DNA extraction (6) was modified and used to detect genetically engineered pseudomonads and a spontaneous deletion derivative in soil using gene probes. Section 5.2, previously published in *Applied and Environmental Microbiology* (Vol. 54, pp. 446-453), describes a most-probable-number DNA hybridization method for enumeration of genetically engineered bacteria in environmental samples.

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5.1 DETECTION OF TWO ENGINEERED PSEUDOMONADS AND A SPONTANEOUS DELETION RECOMBINANT DERIVATIVE IN SOIL USING GENE PROBES

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INTRODUCTION

Until recently, the fate in soil of an engineered DNA sequence was difficult to assess. With developments in DNA isolation protocols, gene probe methodologies can now be employed to monitor the presence of a particular DNA sequence in soil. Most methods using gene probes for detection of specific organisms in environmental samples rely on the isolation of colonies on solid media that are probed by DNA colony hybridization techniques (1,25). However, many microorganisms become dehabilitated in soil (7) and aqueous samples (6,26) so that they are no longer culturable on synthetic media. We recently developed a procedure for the detection of specific microorganisms in soil that overcomes this growth requirement (15). Using this technique, we isolated total bacterial DNA from soil. The purified DNA was then analyzed by Southern blot or slot blot hybridization for the presence of a specific DNA sequence, and hence a specific organism to which that sequence is unique, in the complex mixture of DNA isolated from the soil bacterial community.

In some studies, bacteria engineered with plasmid or transposon markers have been traced in soil (7,8,28). However, recombinant microorganisms released into the environment should not contain foreign genes inserted in this manner because of the potential for horizontal gene transfer (12,13,24). Instead, foreign genes should be stably inserted into the chromosomes of recombinant bacteria. For example, we were able to detect a specific gene, *nptII*, that was engineered into the chromosome of *Bradyrhizobium japonicum* after its addition to soil (15). DNA probe methodology has also been used to monitor the distribution of particular bacterial genes in various environments. For example, Barkay and Olson (2) found a positive correlation between mercury resistance genes (*mer*) and mercury concentration in environmental samples. Similarly, an increase in naphthalene catabolic genes was observed in samples contaminated with this compound (25).

In this study we constructed recombinant strains of *Pseudomonas* sp. as model microorganisms to trace in soil. Pseudomonads are an environmentally and economically important group of organisms. Several are phytopathogens; others seem to promote plant growth. Pseudomonads are also perhaps the most metabolically versatile genus of microorganisms known. Thus, pseudomonads are potentially very important in current and future applied biotechnology and in fact have been proposed for environmental release as hosts for pesticidal and fungicidal agents (22).

Pseudomonas cepacia strain PCO1224 and *Pseudomonas* sp. B8 were used as model organisms in this study. *Pseudomonas cepacia* strains are being used as "benchmark" organisms by the U.S. Environmental Protection Agency (EPA). *Pseudomonas* sp. B8 is an aggressive root-colonizing bacterium that inhibits root growth of winter wheat (11). These strains were engineered to contain a chromosomal insertion of the neomycin phosphotransferase II gene, *nptII*, which encodes resistance to the antibiotic kanamycin. This gene has previously been shown to be essentially nonexistent in the indigenous bacterial community of the soils used in this study (15).

We have employed DNA probe methodology to specifically detect two different species of *Pseudomonas* after their addition to soil. In addition, we found evidence of a spontaneous deletion recombination event in the engineered *Pseudomonas cepacia* strain in soil.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains, strain abbreviations used in the remainder of the text, and plasmids used in this study are listed in Table 1.

Media and Growth Conditions

Pseudomonas strains were routinely grown in Kings B (KB) liquid medium (18). *Pseudomonas cepacia* cultures were grown at 37°C and *Pseudomonas* sp. B8 at 25°C. When incubated together the cultures were grown at 25°C. Antibiotics were added as necessary at the following concentrations: to liquid medium, nalidixic acid, 200 µg/ml; kanamycin, 200 µg/ml;

Table 1. Bacterial Strains and Plasmids[a]

Strain/plasmid Identification	Abbreviation	Relevant Phenotype	Source or Reference
Pseudomonas cepacia	PCO1224 (PC)	Nal^r	R. Olsen
Pseudomonas cepacia	PCO1224-1 (PC-1)	Nal^r, Km^r	This study
Pseudomonas cepacia	PCO1224-2 (PC-2)	Nal^r, Km^r	This study
Pseudomonas sp.	B8 (B8)	Rif^r	L. Elliott
Pseudomonas sp.	B8-1 (B8-1)	Rif^r, Km^r	This study
Escherichia coli	ED8654	Rec$^+$	Borck et al. (1976)
Escherichia coli	N100	Rec$^-$, pro$^-$	M. Rosenberg
	pKC7	Km^r	Rao and Rogers (1978)
	pRL161	Km^r	Elhai and Wolk (1988)
	pRL425	Ap^r, Cam^r Ery^r	J. Elhai, Michigan State University
	pRK2013	Km^r, Tra$^+$	Figurski and Helinski (1979)
	pJJ3	Ap^r	This study
	pJJ5	Ap^r, Km^r	This study
	pBH1	Ap^r	This study
	pJJ6	Ap^r, Km^r	This study

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[a] Abbreviations for antibiotic resistance markers are as follows: Nal, nalidixic acid; Km, Kanamycin; Rif, Rifampicin; Cam, Chloramphenicol; Ery, Erythromycin.

rifampicin, 100 μg/ml; and to solid medium, nalidixic acid, 400 μg/ml; kanamycin, 500 μg/ml; rifampicin, 200 μg/ml; cycloheximide, 200 μg/ml. Minimal medium was also used where stated.

Escherichia coli strains were grown and cultivated in Luria broth (LB) or medium solidified with 1.5% agar. Antibiotics, when used, were added at the following concentrations: for liquid medium, kanamycin, 25 μg/ml; ampicillin, 20 μg/ml; and for solid medium: kanamycin, 50 μg/ml; ampicillin, 40 μg/ml; chloramphenicol, 20 μg/ml.

Soil Inoculation and Sampling

The soil used was Capac loam as described previously (15). Bacterial cultures were grown in KB medium containing appropriate antibiotics to a optical density (OD, 640 nm) of approximately 1 and harvested by centrifugation (10 min at 8000 x *g*). The cells were washed in 0.01 *M* sodium phosphate buffer (pH 7.0) and resuspended in one-half the original volume in the same buffer. Where indicated, cells were starved by incubation in 0.01 *M* sodium phosphate buffer at room temperature for as long as 9 h.

Bacterial suspensions were inoculated into soil by spraying uniformly over the soil surface using a 10-ml syringe with an 18-gauge needle, after which the soil was mixed thoroughly. The soil was moistened to field capacity (approximately 23% moisture) using the cell suspensions; phosphate buffer was used alone for controls. Inoculated soils and uninoculated controls were incubated in plastic bags in the dark at 25°C. At appropriate time intervals, 50-g samples of soil were taken for DNA extraction and 10-g samples were taken for enumeration by plate counting and for dry weight determination. Bacterial populations are expressed as colony-forming units (CFU) per gram dry weight of soil.

Isolation of Bacterial DNA from Soil

Bacterial DNA was isolated from 50-g soil samples by homogenization and differential centrifugation, followed by cell lysis and cesium chloride density gradient centrifugation essentially as previously described (15). The procedure was modified as follows, to reduce the amount of time necessary to complete the extraction process: 1) The soil was subjected to two rounds of blending and centrifugation rather than three rounds; 2) soil bacterial suspensions were washed once in TE buffer; 3) the lysozyme incubation time was decreased to 45 min, pronase incubation time was decreased to 45 min, and pronase incubation time to 30 min; 4) after lysis, cell lysates were immediately prepared into cesium chloride-ethidium bromide density gradients rather than being stored on ice overnight.

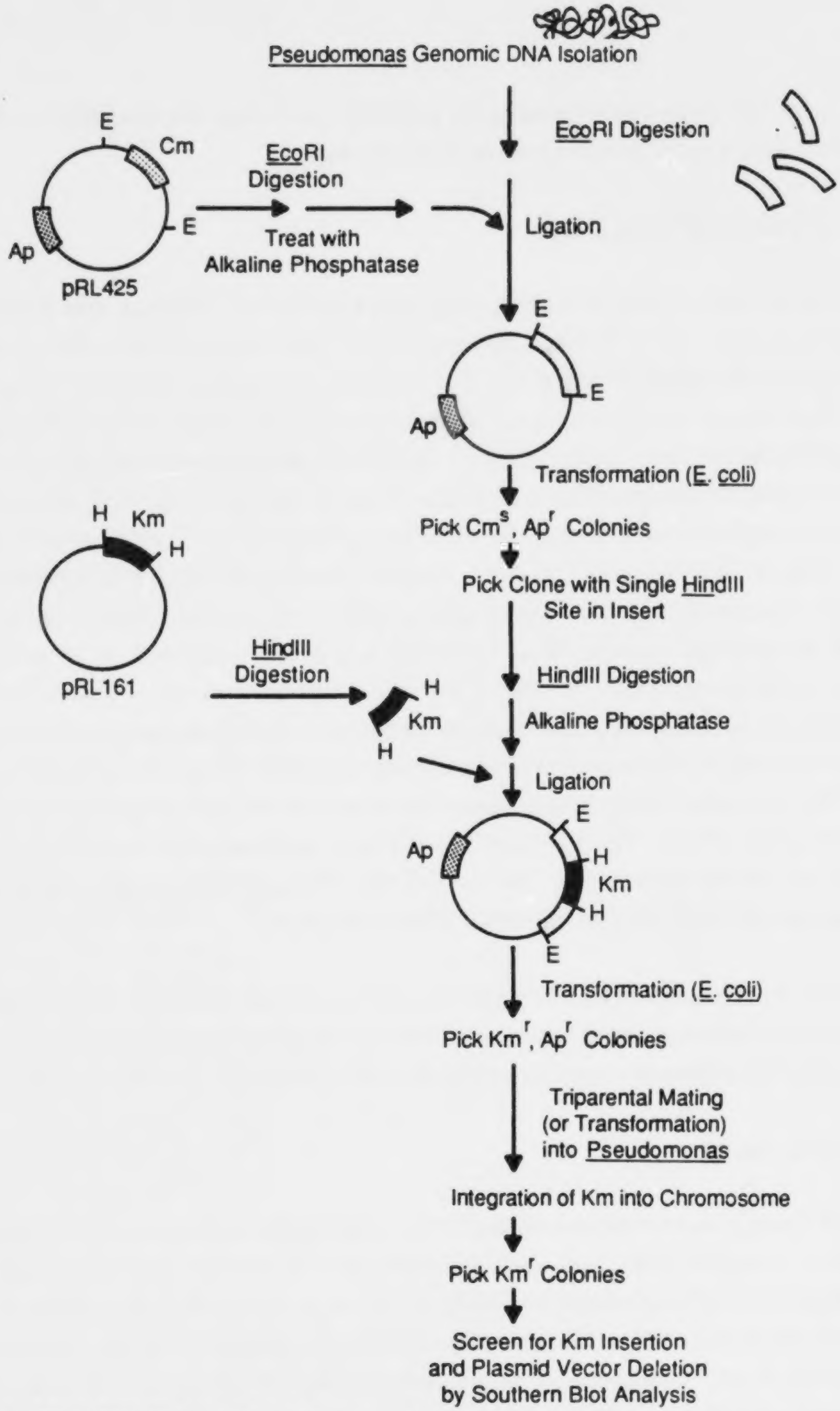
DNA Isolation from Liquid Culture

Bacterial genomic DNA was isolated from liquid culture by a modification of the procedure given by Marmur (21). Bacterial cultures (10 ml) were suspended in 0.1% Sarkosyl (*N*-lauroylsarcosine, Sigma Chemical Co., St. Louis, Missouri) and shaken gently by hand for 2 min. The cultures were then spun at 8000 x *g* for 10 min in Falcon 2059 polypropylene test tubes (Becton Dickinson, Oxnard, California). The cell pellets were resuspended in 2 ml 20% sucrose to which lysozyme (Sigma Chemical Company, St. Louis, Missouri) was added (0.2 ml of 10 mg/ml solution in water); the mixture was then incubated at room temperature for 3 min. Sodium EDTA (40 µl of 250 m*M*) was added followed by 520 µl of 10% Sarkosyl. The mixture was then incubated at room temperature for 45 min. Pronase (Sigma) (50 µl of a 10 mg/ml solution preincubated 30 min at 37°C) was added to the mixture followed by an additional 30 min incubation at 37°C. The lysate was combined with an equal volume of TE saturated phenol, mixed by gentle inversion for 5 min; 2 ml of chloroform was then added. If the cell lysate/phenol mixture was overly viscous, additional TE buffer was added (usually 2 ml). The cell lysate mixture was separated into aqueous and organic phases by centrifugation at 8000 x *g* for 10 min. The aqueous phase was again extracted with phenol/chloroform and then twice with chloroform alone. Where desirable, DNA was precipitated by the addition of two volumes of isopropanol and incubated at -20°C overnight.

Small-scale isolation of plasmid DNA was performed by the method of Birnboim and Doly (3) for *E. coli* strains and by the method of Kado and Liu (16) for *Pseudomonas* strains; large-scale plasmid purification was performed by the method of Clewell and Helinski (5).

Strain Construction

The strategy used for the construction of the recombinant *Pseudomonas* strains is given in Figure 1. Genomic DNA from each pseudomonad was digested with *Eco*RI, and single fragments were randomly cloned into the *Eco*RI site of the mobilizable plasmid pRL425 using standard recombinant DNA techniques (20). Recombinant plasmids were used to transform *E. coli* strain N100. Transformants were selected by their ability to survive on ampicillin-containing medium (ampicillin resestance is conferred by plasmid pRL425), then screened for insertional inactivation of the chloramphenicol resistance gene of plasmid pRL425m, which is


Pseudomonas Genomic DNA Isolation
EcoRI Digestion
E
Cm
E
Ap
pRL425
EcoRI
Digestion
Treat with
Alkaline Phosphatase
Ligation
E
E
Ap
Transformation (E. coli)
Pick Cm^s, Ap^r Colonies
Pick Clone with Single HindIII
Site in Insert
H
Km
H
pRL161
HindIII
Digestion
H
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HindIII Digestion
Alkaline Phosphatase
Ligation
E
Ap
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Km
H
E
Transformation (E. coli)
Pick Km^r, Ap^r Colonies
Triparental Mating
(or Transformation)
into Pseudomonas
Integration of Km into Chromosome
Pick Km^r Colonies
Screen for Km Insertion
and Plasmid Vector Deletion
by Southern Blot Analysis

Figure 1. Procedure for insertion of kanamycin resistance gene (Km) into chromosomes of pseudomonads by homologous recombination.

bordered by*Eco*RI sites, by the loss of chloramphenicol resistance. Insertion of *Pseudomonas* DNA into pRL425 was verified by analysis of plasmid DNA from Amp^r, Cam^S transformants on agarose gels. Plasmids were selected that contained one *Hin*dIII restriction site centrally located in the cloned *Pseudomonas* DNA fragment. The recombinant plasmids pJJ3 (containing *Pseudomonas* sp. B8 DNA), and pBH1 (containing *P. cepacia* DNA) were selected for use in further strain construction steps. The *nptII* gene was isolated as a *Hin*dIII fragment from pRL161 by electroelution from a preparative agarose gel and ligated into *Hin*dIII-digested pJJ3 and pBH1 that had been treated with calf intestinal alkaline phosphatase (Boehringer Manheim) according to the manufacturer's recommendations to prevent recircularization of the plasmid. The ligation mixtures were used to transform *E. coli* N100. Transformants were selected on LB containing ampicillin and kanamycin. The structure of clones that had the *nptII* gene inserted into the *Hin*dIII site was confirmed by analysis of restriction fragment patterns of *Hin*dIII-digested plasmid DNA. The structures of the chosen plasmids (pJJ5 for *Pseudomonas* sp. B8 and pJJ6 for *P. cepacia*) are given in Figure 2.

Triparental Mating

Triparental mating was used to introduce pJJ5 and pJJ6 into *Pseudomonas* sp. B8 and *P. cepacia*, respectively, to allow for homologous recombination into the chromosome of

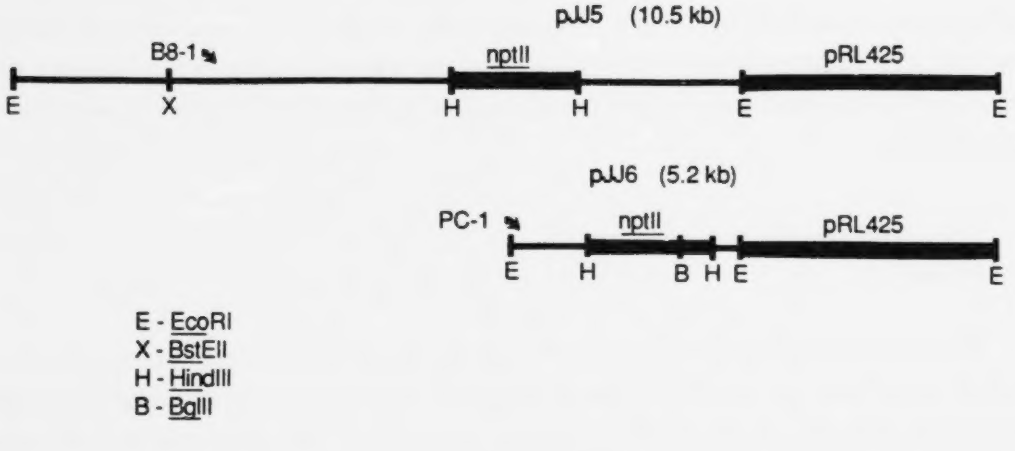

Figure 2. Restriction map of plasmids pJJ5 and pJJ6. Narrow solid lines indicate cloned *Pseudomonas* DNA; wide solid lines indicate *nptII* gene and vector sequences as indicated.

each organism. *E. coli* strains ED8654, containing pRK2013, and N100, containing pJJ5 or pJJ6, were grown separately in LB containing kanamycin, and aliquots were taken so that OD 600 x vol (x) = 0.1. The *E. coli* cultures were centrifuged briefly together in a microfuge tube and washed once with 5% LB. The pseudomonad recipient culture was then added to the same microfuge tube mixture at a vol (x) = OD 600 x vol (x) = 0.176 and collected by centrifugation. The bacterial cell mixture was resuspended in 1 ml 5% LB and added by vacuum filtration to a Millipore filter (0.45-µm pore size) that had been previously washed with sterile distilled water. The filters were placed cell side up on LB agar and incubated overnight at 37°C for *P. cepacia* or 25°C for *Pseudomonas* sp. B8. The filters were then transferred with sterile forceps to LB agar containing nalidixic acid and kanamycin for *P. cepacia* or rifampicin and kanamycin for *Pseudomonas* sp. B8 and incubated at 37° or 25°C, respectively. The filters were then lifted and placed in a sterile test tube containing 0.05 *M* sodium phosphate buffer (pH 7.0) and vortexed to release the bacterial cells. The cell suspension was diluted and plated on the appropriate selective agar medium. Transconjugants were purified by two sequential platings on selective agar. The integration of plasmid DNA into the chromosomes of the transconjugants was verified by Southern blot analysis of genomic DNA.

DNA Hybridization

The single-stranded, ^{32}P-labeled, DNA probe for the *nptII* gene was prepared using the M13 phage-based system described previously (15). Labeling of whole plasmids by nick translation was performed using a nick translation kit (Bethesda Research Laboratories) according to the manufacturer's directions. Southern transfer of DNA and prehybridization and hybridization conditions were as described (26). Colony hybridization was performed as described (14).

RESULTS

Pseudomonas cepacia PCO1224 and *Pseudomonas* sp. B8 were engineered to contain a chromosomal insertion of the *nptII* gene that encodes resistance to the antibiotic kanamycin, resulting in recombinant strains PC-1 and B8-1, respectively. The strategy for insertion relied on homologous recombination between plasmids carrying cloned fragments of *Pseudomonas* chromosomal DNA containing the *nptII* gene insertion and the homologous chromosomal sequences. Recombinants were detected by resistance to kanamycin. It was not possible to efficiently screen for the absence of the plasmid vector, pRL425, by antibiotic resistance markers, because the antibiotic selection on this plasmid vector is ampicillin resistance and both *Pseudomonas* strains were naturally resistant to ampicillin. Therefore, to screen for the presence of pRL425 sequences, the recombinants were analyzed by Southern blot analysis of isolated genomic DNA using ^{32}P-labeled pRL425 and pKC7 (which contains the *nptII* gene but has no homology to pRL425) as probes. In each case pRL425 sequences were found to be present in the chromosome of the recombinants, indicating that the vector had not been eliminated.

Because the ColE1 origin of replication of pRL425 cannot replicate in pseudomonads, the entire recombinant vectors (pJJ5 or pJJ6) must have been inserted into chromosomes of the *Pseudomonas* strains by a single crossover event (refer to Figure 3). To verify this hypothesis, the genomic DNA of each of the transconjugants was digested with a restriction enzyme that cuts only once in the recombinant vector (*Bgl*II for pJJ6 and *BstE*II for pJJ5) (see Figure 2). The DNA fragments were size fractionated on a 1% agarose gel, transferred to a cellulose nitrate filter, then probed with ^{32}P-labeled pJJ5 or pJJ6. In each case two bands were observed (data not shown), indicating that the entire plasmid had been integrated into the chromosomes of each of the pseudomonads. We tried to detect spontaneous conversion of the single crossover structure to a double crossover structure (see Figure 3) by incubation in nonselective medium and screening by colony hybridization for spontaneous deletion of the integrated vector sequence using pRL425 as a probe, but were unsuccessful after screening 600 individual colonies.

Strains PC-1 and B8-1 were inoculated singly or together into nonsterile soil samples. After 1 day of incubation, the soil bacterial DNA was extracted, digested with *Cla*I, size fractionated by electrophoresis, and then transferred to cellulose nitrate and hybridized with the ^{32}P-labeled probe for the *nptII* gene (Figure 4). A number of restriction enxymes were screened to find one that did not cut in either pJJ5 or pJJ6 to allow each of the two *Pseudomonas* species to be detected as a single band of a discrete size for each organism. The restriction enzyme, *Cla*I, was found to be suitable, and this enzyme was subsequently used to digest DNA isolated both from soil and from liquid cultures. DNA from soil inoculated with either strain individually when probed as described, generated a band of identical size when compared to the pseudomonad genomic DNA control (Figure 4, lanes A, B, C and F, G, H). The two pseudomonads could easily be distinguished from each other in the same soil sample on the basis of the difference in size of the *Cla*I restriction fragments containing the *nptII* gene insertions (Figure 4, lanes D and E).

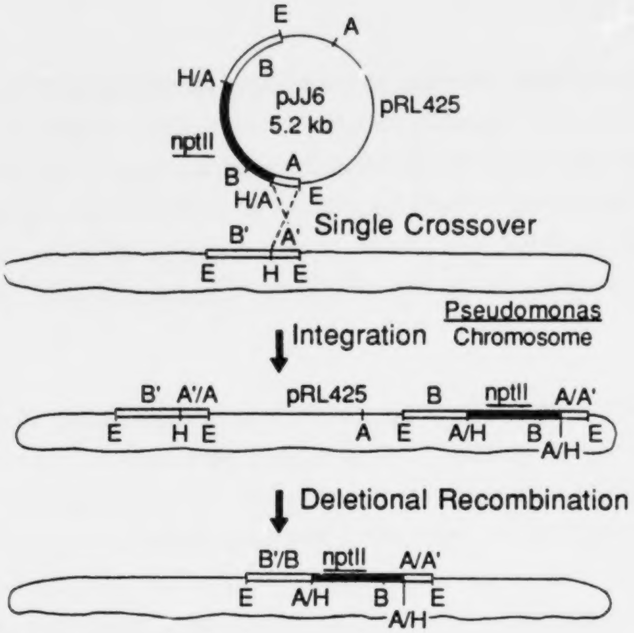
E
A
H/A
B
pJJ6
5.2 kb
pRL425
nptII
A
B
H/A
E
B'
A'
Single Crossover
E
H E
Pseudomonas
Chromosome
Integration
B' A'/A
pRL425
B
nptII
A/A'
E
H E
A E
A/H
B
E
A/H
Deletional Recombination
B'/B
nptII
A/A'
E
A/H
B
E
A/H

Figure 3. Graphic representation of integration of pJJt into *Pseudomonas cepacia* chromosome and subsequent deletion recombination of integrated pRL425 sequences. Restriction sites are designated by small capital letters: E, *Eco*RI; A, *Alu*I; H, *Hin*dIII, B, *Bgl*II. Large capital letters represent *P. cepacia* chromosomal fragments: A, B, A', B'. Neomycin phosphotransferase II gene insertion, *nptII*.

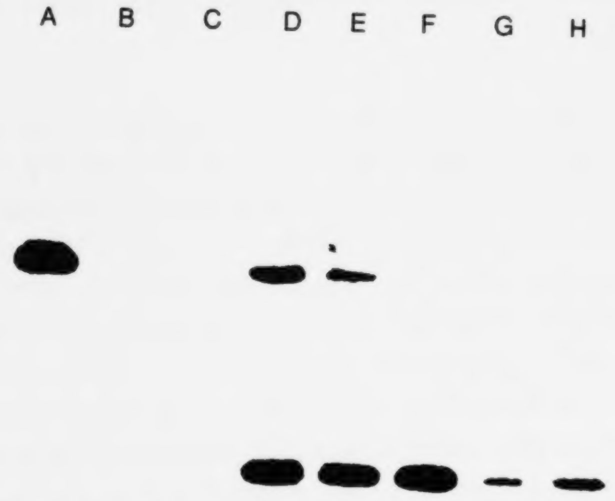

Figure 4. Autoradiogram from Southern blot of *Cla*I-digested total bacterial DNA isolated from soil samples that were inoculated with PC-1 and B8-1. Lanes A and H contain genomic DNA isolated from liquid cultures of B8-1 and PC-1, respectively. Lanes B through G contain DNA isolated from soil inoculated with B8-1 alone (B and C); with PC-1 alone (F and G); or with B8-1 and PC-1 (D and E). Lower band in lanes D through F represents a deletion recombination derivative of PC-1.

An unanticipated result was the appearance of an anomalous band when DNA isolated from soil that was inoculated with PC-1, either singly or with B8-1, was digested with *Cla*I and probed with the *nptII* gene probe (Figure 4, lanes D, E, F, G). Comparison to DNA size standards of known molecular weight indicated that this band was of the size predicted for a double-crossover recombination event, resulting in integration of only the cloned *Pseudomonas* DNA fragment containing the *nptII* gene (refer to Figure 3). Genomic DNA isolated from liquid pure culture of this organism did not contain this anomalous band, indicating that the deletion recombination event occurred in soil.

To determine whether the deletion recombination event occurred in liquid culture before addition to soil or only after incubation in soil, colony hybridization was used to screen for colonies that did not contain pRL425 vector sequences. Colonies plated from the original inoculum were compared to colonies isolated from soil, and from cultures incubated at the same time in minimal medium at 4°, 25°, or 40°C (Table 2). After 1 day of incubation, the soil and minimal medium cultures were plated onto KB selective medium containing kanamycin and nalidixic acid. Colonies were picked, inoculated onto KB selective agar plates in an ordered array, and analyzed by colony hybridization for the presence of pRL425 vector sequences by

Table 2. Frequency of Detection of Deletional Recombinants After Incubation of PC-1 in Soil or Liquid Cultures

Incubation Time (Days)	Condition	Incubation Temperature (°C)	Frequency (%)[a]
0	Inoculum	37	0
1	Soil	25	1
1	Minimal media	4	1
1	Minimal media	25	0
1	Minimal media	40	1.5

---

[a] Frequency is expressed as the percent of total colony isolates on KB selective medium that do not hybridize to the pRL425 probe by DNA colony hybridization. The data presented represent averages from four replicates (100 colonies/replicate) for soil and original inoculum, and two replicates for minimal media cultures. In one instance a frequency of 4% was observed after incubation in soil.

probing with ^{32}P-labeled pRL425 (Figure 5) The recombination event was only detected in colonies isolated from soil, and in cultures stored at 4° or 40°C, but not those stored at room temperature (Table 2).

Three colonies that did not hybridize to the pRL425 probe were further analyzed by restriction digest analysis using Southern blots. Data from the analysis of DNA from putative deletional recombination derivative colonies isolated from soil are given in Figure 6A. The three isolates did not hybridize to the pRL425 vector compared to a control with integrated pRL425. The same samples when probed with the ^{32}P-labeled *nptII* gene probe resulted in a single band of the size predicted for precise deletion of the pRL425 vector (Figure 6B, lanes A, C, and D).

PC-1 genomic DNA was digested with *Eco*RI and probed with various ^{32}P-labeled probes on a Southern blot to distinguish the remaining chromosomal fragment that did not participate in the initial crossover event (Figure 7). The band in lane A, 2.7 kb, corresponds to pRl425 DNA and in lane B, 2.3 kb, to the B + *nptII* + A/A' DNA segment (Figure 3). The broad band in lane C consists of the closely positioned 2.7- and 2.3-kb bands seen in lanes A and B, respectively. The arrow indicates the additional (1.0) chromosomal fragment that did not participate in the original crossover event, corresponding to the B' + A'/A DNA fragment.

Frequencies less than 10^{-2} cannot be easily detected by the colony hybridization method because it is not practical to screen large numbers of colonies; thus, bacterial DNA was isolated from soil and from liquid cultures and analyzed by Southern blot analysis. In most cases the deletion was only observed in DNA isolated from soil. However, in one instance the fragment representing the deletion recombination was observed in liquid cultures incubated at room temperature, and in that particular experiment it was also observed in the original inoculum (not shown).

Results from additional experiments indicated that the freshness of the soil, growth stage of the culture, period of starvation, or presence or absence of nalidixic acid in the growth medium did not affect the frequency of recombination at the level of detection possible with colony hybridization. An experiment to determine whether the proportion of recombinants increased with an increased incubation time in soil was determined by direct comparison of DNA isolated after various times of incubation. The relative proportion of the single crossover band to recombinant band remained constant (data not shown). There was, however, a decrease in overall hybridization intensity for samples incubated 2 weeks as compared to 1 week.

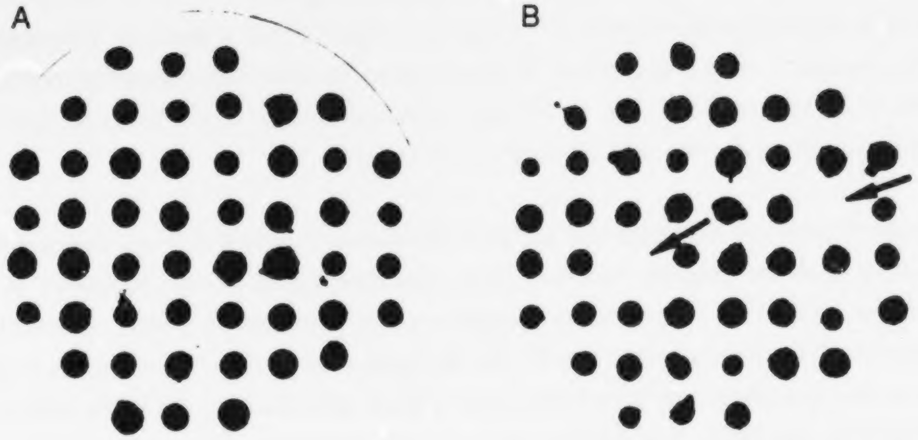

Figure 5. Autoradiogram from colony hybridization analysis using [^{32}P]-labeled pRL425 as probe. Colonies isolated from original inoculum (A); colonies isolated from soil (B). *Arrows* indicate colonies that did not hybridize to pRL425, presumably because of its loss by deletion.

DISCUSSION

This study demonstrates the ability of gene probes to detect genomic rearrangements in organisms inoculated into soil. In addition, gene probes were shown to be able to detect and distinguish between two different *Pseudomonas* species present in a single soil sample by the detection of discrete bands of a predicted size in the complex mixture of DNA isolated from nonsterile soil. We used both Southern blot and colony hybridization methodologies to detect deletion recombinant derivatives of PC-1 in soil. Southern blots were advantageous in that they were capable of detecting recombinants in soil at frequencies lower than could be practically determined by colony hybridization. Colony hybridization was more useful, however, for determining frequency values, because a numerical value was difficult to obtain directly from an autoradiograom resulting from a Southern blot.

Soil is thought to stress many bacteria after prior culturing in liquid media (7). In this study, the number of deletion recombinants were similar in soil and in minimal medium liquid cultures incubated at 4° or 40°C. These extreme temperature conditions are far from the optimum for growth of PC-1, and thus may also cause stress-induced recombination events. We have shown that these pseudomonads do not grow in soil under the conditions used in this study. Instead, there is a steady decline in numbers dduring several weeks incubation until an equilibrium number (approximately 10^6 cells/g dry weight of soil) is reached. This study may represent a case in which conditions of stress caused genome rearrangements resulting in deletion of inessential DNA. The *nptII* gene is also nonessential in soil but is not flanked by homologous regions as is the pRL425 DNA (refer to Figure 3).

In one experiment, Southern blot analysis showed that the same deletion had occurred in DNA from both the original inoculum and a saturated liquid culture incubated at room temperature. We do not believe that the delection recombination event always occurred before introduction of the strain to soil. For example, in Figure 4 the genomic DNA band is of greater intensity than one of the bands for DNA isolated from soil; however, the lower band is only observed in the soil DNA. It is possible that prolonged incubation of saturated cultures, and/or stationary or log-death phase, similarly stress bacteria, as indicated in the experiment in which the deletion was observed in the original inoculum and in cells stored at room temperature. This particular experiment was at a later date, so it is possible that the strains were becoming more unstable with increased subculture and storage. It is also possible that that clones initially scored as stable could become unstable after subculture. This phenomenon has been described as delayed marker loss.

It is not clear why the whole-plasmid insertion was less stable in PC-1 than in B8-1. Lloyd et al (19) found that nalidixic acid mutants have a higher frequency of deletion recombination because of the nature of the mutation affecting DNA gyrase. This may explain, in part, why the deletion was only observed in the nalidixic acid-resistant pseudomonad and not in strain B8-1. It has been postulated (17) that larger regions of homology improve the potential for recombination. However, in strain B8-1 the flanking regions of homology were larger.

The principle of detecting genomic rearrangements by restriction fragment-length polymorphism applies whether the rearrangement is within the genome of an individual organism or results from gene transfer between organisms. Thus, the method should be equally useful in assessing horizontal gene transfer in nature.

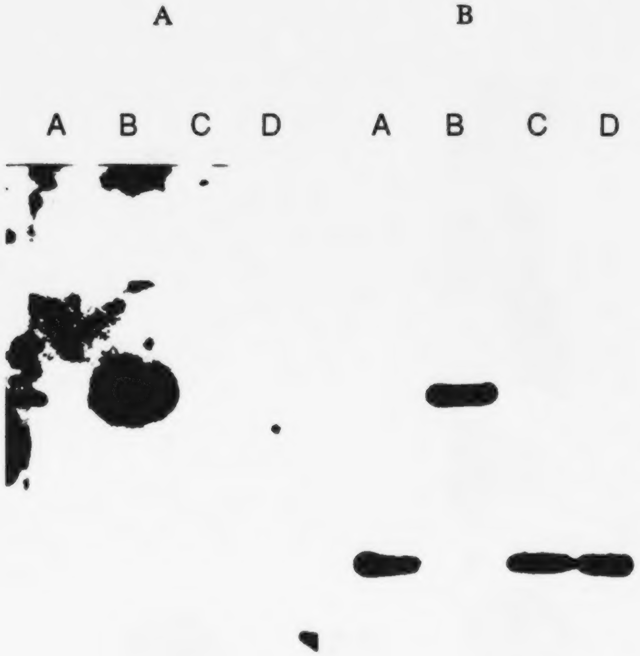

Figure 6. Autoradiogram from Southern blot after hybridization with [^{32}P]-labeled probes to *Cla*I-digested DNA isolated from putative deletion recombinants. Duplicate filters were probed with either pRL425 (left) or the *nptII* gene (right). Lanes A, B, and C contain DNA from colonies that did not hybridize to pRl425 by colony hybridization. Lane B is a control with DNA from a colony that did hybridize to pRL425.

Figure 7. Autoradiogram obtained after Southern transfer of *Eco*RI-digested PC-1 DNA hybridization with various probes. PC-1 genomic DNA (6 μg) was digested with *Eco*RI and size fractionated by agarose gel electrophoresis. The Southern blot was probed with pRL425 (A), with single-stranded *nptII* (B), or with pBH1 (C).

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5.2 ENUMERATION OF Tn5 MUTANT BACTERIA IN SOIL BY MPN-DNA HYBRIDIZATION AND ANTIBIOTIC RESISTANCE*

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SUMMARY

Investigations were made into the utility of DNA hybridization in conjunction with a microdilution most-probable-number (MPN) procedure for the enumeration of *Rhizobium* spp. and *Pseudomonas putida* in soil. Isolates of *Rhizobium* spp. and *P. putida* carrying the transposon Tn5 were added to sterile and nonsterile Burbank sandy loam soil and enumerated over time. Soil populations of rhizobia were enumerated by colony hybridization, MPN-DNA

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hybridization, plate counts, plant infectivity MPN, and fluorescent antibody counts. Population values compared well for all methods at 5 and 30 days after addition of cells, although the fluorescent antibody method tended to overestimate the viable population. In nonsterile soil, MPN-DNA hybridization enumerated as few as 10 *P. putida* Tn*5* cells per gram of soil and 100 *R. leguminosarum* biovar *phaseoli* Tn*5* cells/g of soil, and should have utility for following the fate of genetically engineered microorganisms released to the environment. Among the Km^r isolates containing Tn*5*, approximately 5% gave a dark, more intense autoradiograph when probed with [^{32}P]pGS9 DNA, which facilitated their detection in soil. Hybridization with a pCU101 probe (pGS9 without Tn*5*) indicated that donor plasmid sequences were being maintained in the bacterial chromosome. Transposon-associated antibiotic resistance was also utilized as a phenotypic marker. Tn*5* vector-integrate (VI) mutants were successfully enumerated at low populations (10 to 100 cells/g of soil) in soil by both phenotypic (Km^r) and genotypic (DNA probe) analysis. However, determination of the stability of Tn*5* or Tn*5* and vector sequences in the bacteria is necessary.

INTRODUCTION

The application of genetically engineered microorganisms (GEMs) for waste treatment, agronomic crop production, and mineral and energy-product recovery will likely require release of the altered organisms to the environment. Numerous concerns have been raised about potential hazards to the ecosystem from the release of GEMs (16,6,28,9). Assessing the risks involved in the release of GEMs requires tracking the environmental fate of the organisms to determine their survival, growth, and dissemination (2). However, technologies to improve the detection and enumeration of microorganisms in the environment have been slow to develop.

A variety of standard methods are used to detect and enumerate microorganisms in the environment, including direct plating, enrichment cultures, identification of characteristic cell components (e.g., fatty acids, proteins, rRNAs), direct microscopy, serology, immunofluorescence, and immunoradiography. The advantages and disadvantages of each, which have been the subject of much discussion, are reviewed by McCormick (25). One of the more recent developments in the environmental detection and enumeration of specific microorganisms is based on the expression of cloned *E. coli lacY* and *lacZ* genes in *Pseudomonas fluorescens* (10). One disadvantage to this method is that it is limited to microorganisms without the intrinsic ability to utilize lactose. DNA probes have been used to detect *Salmonella* spp. (12) and *Yersinia* spp. (18) in foods, and to detect specific catabolic genes in sediments (31). Advantages of DNA probes are high specificity (17) and the potential for detection of gene or plasmid transmission.

Spontaneous antibiotic-resistant mutants have been used extensively for following populations of microorganisms in soils and the rhizosphere (37,13,39), and will likely continue to have utility as a selective marker in microbial ecology. However, the environmental competitiveness of the organisms may be altered by biochemical impairment associated with the mutation (35). Transposable elements are recognized as valuable tools for genetic manipulations and characterizations (21), but only recently has transposon-associated antibiotic resistance been used as a selective marker in microbial ecology experiments (36). Transposon Tn*5* insertions have several advantages over spontaneous mutants in that the mutation can be well characterized with respect to location in the bacterial chromosome, multiple insertions occur at low frequencies, insertions occur at a large number of sites (increasing the likelihood of obtaining a mutant which is not biochemically impaired with respect to the parent), and a region of homology for DNA hybridization is available. The Km^r phenotype associated with the Tn*5* insertion has been shown to be stable after growth for more than 50 generations on nonselective media (36), but it is not known to what extent transposons are stable in bacteria growing in soil.

In this paper we compare several methods for detecting bacteria in soil and assess the utility of the transposable element Tn*5* as a phenotypic marker in microbial ecology experiments. In addition, a quantitative DNA hybridization procedure for detection of unique genotypes in soils using most-probable-number (MPN) analysis is presented, which may be applicable to the detection and enumeration of genetically engineered microorganisms intended for release to the environment.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial recipients for transposon mutagenesis were *Pseudomonas putida* ATCC 23973 (harboring the biodegradative plasmid, pWWO, that codes for the catabolism of toluene), obtained from G. Sayler (University of Tennessee), *Rhizobium leguminosarum* biovar *viceae* (strain IP), and *R. leguminosarum* biovar *phaseoli* (strain Kim-5). The isolate used as a conjugative transposon donor and the source of DNA for [^{32}P] probes was *Escherichia coli*

MV12 (Leu$^-$ Thr$^-$ Thi$^-$ Thy$^-$ Trp$^-$) transformed with the suicide plasmid vector pGS9 (Cm^r and Km^r) containing the transposon Tn*5* (33), from V. Iyer (Carlton University). Plasmid pCU101 (Cm^r), which is identical to pGS9 but without Tn*5* sequences, was maintained in *E. coli* HB101.

Media, Cultural Conditions, and Matings

Stock cultures of all bacterial isolates were maintained in a glycerol-medium mixture (1:1) frozen at -80°C. *Pseudomonas putida* was routinely cultured on Kings medium B (KB) broth or agar (20), *Rhizobium* strains on yeast extract mannitol (YEM) broth or agar (38), and *E. coli* on Luria-Bertani (LB) medium (23). *Pseudomonas putida* and the *Rhizobium* strains were grown at 27°C and *E. coli* at 37°C. For bacterial matings, log-phase cultures of donor and recipient cells were centrifuged in an Eppendorf microfuge for 5 min and washed twice in sterile deionized water before resuspension to obtain 10^9 cells/ml. Donor and recipient cells (10^8/0.1 ml each) were mixed and placed on sterile 0.45-µm nitrocellulose membrane filters on LB agar plates and incubated for 24 h at 27°C. Selection for transconjugants was made on either *Pseudomonas* minimal salts (PMS) agar (14) or *Rhizobium* minimal salts (RMS) containing, per liter: 15 g agar; 10 g mannitol; 1.0 g K_2HPO_4; 1 g KH_2PO_4; 0.5 g NH_4Cl; 0.25 g Na_2SO_4; 0.25 g $MgCl_2 \cdot 7H_2O$; 0.1 g $CaCl_2 \cdot 2H_2O$; 0.01 g $CaCO_3$; 10 mg $FeCl_3 \cdot 6H_2O$; and 1 mg each biotin and thiamine with 50 µg/ml or 100 µg/ml of kanamycin for *Pseudomonas* and *Rhizobium*, respectively.

Plasmid DNA and Probe Preparation

Escherichia coli MV12 was cultured in LB broth with 50 µg/ml each chloramphenicol and kanamycin (Sigma Chemical Company, St. Louis, Missouri), which selected for the plasmid pGS9 and maintenance of the transposon Tn*5*. Plasmid DNA preparations were performed according to the method of Maniatis et al. (23) by alkaline lysis with an additional chloroform extraction followed by isopropanol precipitation of DNA. Purified plasmid DNA for use as probes was prepared by ultracentrifugation of plasmid preparations on CsCl (1.0 g/ml) density gradients with 0.5 mg/ml of ethidium bromide at 55,000 rpm for 17 h at 20°C followed by 1 h at 45,000 rpm at 20°C. Plasmid bands were removed and extracted four times with isopropanol:5 *M* NaCl (1:1 vol/vol) in 10 m*M* Tris pH 8.0 buffer to remove ethidium bromide. DNA was recovered by ethanol precipitation. ^{32}P-labeled DNA probes were made using a nick-translation kit (Bethesda Research Laboratories, Gaithersburg, Maryland) following the suggested protocol. [^{32}P]dCTP (3000 Ci/mmol; New England Nuclear Corp., Boston, Massachusetts) was used as the labeling nucleotide. The labeled probe was separated from unincorporated nucleotides using a spun-column procedure (23), and the activity was quantified by liquid scintillation counting.

Rapid visualization of plasmid DNA was accomplished by the method of Eckhart (11) with the following modifications. After centrifugation of cells and removal of medium, the pellet was resuspended in 0.1% Sarcosyl (*N*-lauroylsarcosine; Sigma) in 50 m*M* Tris:25 m*M* EDTA (pH 8.8) and incubated at room temperature for 10 min in 1.5-ml microfuge tubes. Cell suspensions were repelleted and resuspended in lysozyme mixture and incubated at room temperature for 10 to 15 min, followed by gentle mixing with the SDS (sodium dodecyl sulfate; Sigma) mixture and incubation for 10 min. Proteinase K (5 µl, 5 mg/ml; International Biotechnologies Inc., New Haven, Connecticut) was added to the microfuge tubes and mixed gently. The suspension was then transferred to the wells of a 0.7% agarose gel and sealed with molten agarose. DNA was electrophoresed for 1 h at 35 V and then for 6 h at 100 V. Transfer of DNAs visualized by this procedure onto GeneScreen Plus (New England Nuclear) was performed according to the manufacturer's suggested protocol.

Enumeration of Soil Populations

Soils used for Tn*5* mutant recovery experiments were a Burbank sandy loam and a Palouse silt loam. Where sterile soil was used, soil was autoclaved (121°C, 18 psi) on 3 consecutive days for 30 min. For enumeration of Tn*5* mutants from soil using antibiotic resistance as a selective marker, cultures of *R. leguminosarum* and *P. putida* containing Tn*5* were enumerated by hemacytometer and added to 5.0 g of air-dried Burbank and Palouse soils at 10^6, 10^4, 10^2, and 0 cells/g of soil in 25-ml centrifuge tubes. Sterile deionized water was added to bring the water potential of both soils to -0.03 MPa. Soils with cells were thoroughly mixed by vortexing for 2 min and incubated at 4°C for 1 h. Sterile broth was added, and the contents were serially diluted and plated in triplicate. For *Rhizobium*, aliquots of 100 µl from each dilution were spread onto YEM agar containing 100 µg/ml each of kanamycin sulfate, cycloheximide, and pimarcin (Sigma). Aliquots from the *P. putida* treatments were spread onto agar plates selective for fluorescent pseudomonads (30) amended with 100 µg/ml of kanamycin (Km). Plates were incubated at 27°C for 1 to 4 days before enumeration. For enumerations by DNA colony hybridization, colony blots from appropriate dilutions were performed with Whatman no. 541 paper according to the procedure described by Gergen et al. (15). Filters were dried completely at room temperature before hybridization.

Enumeration of *R. leguminosarum* and *P. putida* Tn*5* mutants added to 5.0 g of Palouse and Burbank soils at levels ranging from 10 to 10^6 cells/g of soil were made by MPN-DNA hybridization. After mixing soils and cells and incubating as described, 24.0 ml of sterile YEM broth containing the antibiotics previously listed or unamended KB broth were added to 5 g of soil to provide an initial soil:solution ratio of 1:5 (wt/vol). Spontaneous mutants of *R. leguminosarum* biovar *phaseoli* (Kim-5), resistant to 100 µg/ml rifampicin (Sigma), were selected to provide an additional marker. Five 277.5-µml aliquots were removed and added to five wells in a sterile microdilution plate. A serial 1:10 dilution was made by pipetting 27.5 µl of the initial aliquots to the next set of five wells containing 250 µl of sterile broth with antibiotics to obtain a total volume of 277.5 µl. Dilutions were made to extinction. Microdilution plates were incubated at 27°C for 3 to 4 days. The entire contents of each well (250 µl) was quantitatively transferred to individual wells of a microsample filtration manifold (Schleicher & Schuell, Inc., Keene, New Hampshire), taking care to avoid disturbing and transferring sedimented soil particles, and filtered onto GeneScreen Plus. Cells were lysed, DNA was denatured and fixed by passing two volumes (250 µl each) of 0.5 *N* NaOH through the individual wells, followed by identical treatment with 1 *M* Tris pH 7.5, and filters were air dried. After DNA-DNA hybridization and autoradiography, positive blots indicating the presence of target DNA were scored for each dilution, and the MPN value for a five-replicate 10-fold dilution series calculated (1). Uninoculated soils served as controls to discern any background homology or nonspecific binding of the probe.

DNA-DNA Hybridization

GeneScreen Plus filters (New England Nuclear) were placed back to back in a heat-sealable plastic bag with the manufacturer's suggested prehybridization solution and incubated for 6 to 8 h at 65°C. Whatman 541 filter colony blots (VWR, Seattle, Washington) were saturated with 6x SSC (sodium chloride-sodium citrate) (1x SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate) and washed for 1 to 2 h in a solution containing 50 m*M* Tris pH 8, 1 *M* NaCl, 1 m*M* EDTA, and 0.1% SDS at 37°C with constant agitation to remove cell debris. Prehybridization was done as previously described for GeneScreen Plus with 5x SSC substituting for the prehybridization solution. Salmon sperm carrier DNA and ^{32}P-labeled DNA probe were denatured by heating in boiling water for 10 min and added to plastic bags containing filters and solutions at 150 µg/ml and 10^5 dpm/ml hybridization solution, respectively. Plastic bags were resealed and incubated at 65°C for 12 to 14 h with agitation.

Filters were removed from bags and washed according to manufacturer's directions for GeneScreen Plus filters. Whatman 541 colony blots were washed three times in 2x SSC at 65°C (20 min each), followed by one wash in 0.2x SSC at 55°C for 20 min. Autoradiography was carried out at -70°C using Kodak XAR-5 x-ray film.

Stability of Tn*5* Mutants in Soil

The stability of *P. putida* and *R. leguminosarum* Tn*5* mutants was tested in sterile Burbank soil. Bacteria were added to 10 g sterile soil in 50-ml centrifuge tubes to obtain a density of approximately 200 cells/g dry wt. Soil water potential was adjusted to approximately -0.03 MPa with sterile water, and the tubes were incubated at 22°C. After 7 days, bacterial populations were enumerated by plate counts on KB and YEM for *P. putida* and *R. leguminosarum*, respectively, 0.1-g samples of soil were transferred to new tubes containing sterile Burbank soil, and the cycle was repeated twice. Individual colonies were picked randomly from plates used to enumerate populations and grown in nonselective medium (no kanamycin) in microdilution plates. Sensitivity to kanamycin was tested by replica plating from the microdilution plates to agar medium containing 50 μg/ml kanamycin. The presence of donor plasmid sequences was determined by spot-blot DNA hybridization with ^{32}P-labeled pCU101 (PGS9 without Tn*5*, as described previously) of individual colonies grown in microdilution plates.

Fluorescent Antibody (FA) Counting

Procedures for preparation of antibodies and fluorescein isothiocyanate conjugates were described previously (4,32). Soil (1:10 dilution) was dispersed for 5 min by shaking with 1 drop each of Tween 80 and antifoam AF71 and then flocculated with 1% $CaCl_2$ for 1 h. Supernatant was diluted, when necessary, and passed through a 25-mm-diameter, 0.22-μm-pore-size Nuclepore (Nucleopore Corp., Pleasanton, California) filter previously stained with Irgalan black (4). Filters were treated with a gelatin-rhodamine conjugate (4), and then with the specific fluorescein isothiocyanate conjugate to Kim-5. Positive colonies from 20 to 30 fields were counted with a Zeiss epifluorecense microscope.

Plant Infectivity

Most-probable-number plant infectivity enumerations for *R. leguminosarum* biovar *phaseoli* were conducted on soil dilutions with surface-sterilized seeds of *Phaseolis vulgaris* cultivar Viva Pink pregerminated for 5 days in sterile growth pouches (38); MPN values were determined by the presence or absence of nodules in five replications per soil dilution.

RESULTS

Isolation and Characterization of Tn*5* Mutants

In matings between the *E. coli* MV12 donor and *P. putida*, Km^r transconjugants were obtained at a frequency of about 10^{-5} per recipient cell, whereas for *R. leguminosarum* strains the frequency was approximately 10^{-6}. No attempt was made to select or quantify auxotrophs to avoid the use of mutants crippled in biosynthetic pathways in recovery experiments. Colony blots of Km^r mutants, probed with ^{32}P-labeled pGS9 DNA to confirm the presence of Tn*5*, revealed signals of two intensities when autoradiographed. Those isolates with the more intense (dark) signal comprised approximately 5% of the total Km^r mutants for both *Rhizobium* spp. and *P. putida*. Light and dark isolates were subjected to agarose gel electrophoresis, which revealed that pGS9 was not being maintained extrachromosomally (Figure 1). Southern analysis of this gel (Figure 1) using ^{32}P-labeled pGS9 DNA as a probe shows that Tn*5* is located in the chromosome of both light and dark isolates and not on the TOL plasmid in *P. putida* or indigenous plasmids in *Rhizobium* spp.

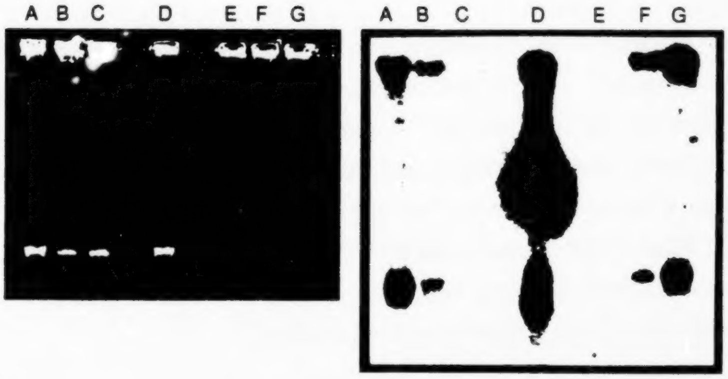

Figure 1. Eckhardt-type agarose gel (left) and autoradiograph of Southern transfer of the gel probed with ^{32}P-labeled pGS9 DNA (right) of *R. leguminosarum* bv. *viceae* IP Tn*5* isolates (lanes A, B) and *R. leguminosarum* bv. *viceae* IP wild type (lane C) with indigenous plasmids, *E. coli* WA803 with pGS9 (lane D), *P. putida* wild type with TOL plasmid (lane E), and *P. putida* Tn*5* isolates (lanes F, G).

Dot-blot analysis of *P. putida* Tn*5* isolates was conducted with 20 ml of culture containing approximately 10^9 cells/ml to obtain about the same amount of DNA for each dot. Hybridization of the blot with pCU101 probe revealed that *P. putida* mutants 1 and 3 contained plasmid vector sequences as evidenced by the relatively intense autoradiograph (Figure 2). Mutants 2 and 3 showed slight hybridization because of the small amount of Tn*5* DNA present in pCU101.

Stability of Tn*5* Mutants in Soil

The stability of Km^r mutants containing pGS9 sequences (vector integrates) was examined by screening individual colonies for both Tn*5* and donor plasmid sequences after growth of the bacteria in sterile Burbank soil. These Tn*5* vector-integrate mutants (Tn*5* VI) were chosen for soil recovery studies because of the increased sensitivity of their detection by DNA hybridization over those isolates containing Tn*5* sequences alone. After growth for 33 generations, none of 184 colonies of *R. leguminosarum* showed sensitivity to 100 µg/ml kanamycin. However, 22 of 184 colonies no longer hybridized with ^{32}P-labeled pCU101 DNA. Of the 181 *P. putida* colonies, 1 was found to be sensitive to 100 µg/ml kanamycin after 34 generations in soil while only 2 failed to hybridize with pCU101 probe. The colony that was Km^s also failed to hybridize with the pCU101 probe, indicating loss of both Tn*5* and suicide plasmid sequences.

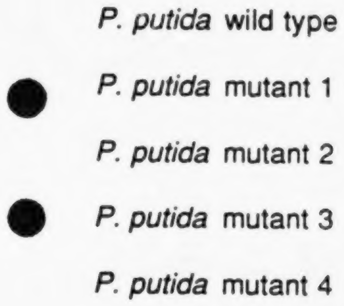

Figure 2. Dot blot of 20 µl of *P. putida* wild type and mutant cultures probed with ^{32}P-labeled pCU101 DNA. *P. putida* mutants 1 and 2 correspond to those in lanes G and F, respectively, in Figure 1.

Detection of Bacteria Incubated in Soil by MPN-DNA Hybridization

A MPN-DNA hybridization procedure was developed for detecting and enumerating specific bacterial genotypes in soils. The populations of *P. putida* Tn*5* in sterile and nonsterile Burbank soil during a 27-day incubation period are shown in Figure 3. The population in sterile soil increased rapidly to more than 10^7 MPN/g soil then declined. In nonsterile soil the bacteria did not grow and populations declined by about 1 log unit during the incubation period. The autoradiograph from which the MPN values for day 27 were calculated for *P. putida* inoculated into sterile and nonsterile soil is shown in Figure 4. In the sterile soil, *P. putida* was diluted to extinction by the sixth row of wells ($10^{-5.7}$), whereas in the nonsterile soil only three of the five wells in the first dilution probed positive.

To ensure against false-positives caused by nonspecific binding to soil particles or homology of probe DNA with any sequences in indigenous soil bacteria, proper controls are necessary. Controls include uninoculated sterile and nonsterile Burbank soil as well as positive (confirmed Tn*5* mutants) and negative (wild-type) pure culture controls. Pure culture controls are shown in column 11 of Figure 4. Any nonspecific binding of probe to uninoculated sterile or nonsterile soil controls was not sufficient to expose film (7 h). In addition, there is no cross-reactivity of the pGS9 probe with either *E. coli* C-1a or the *P. putida* wild type.

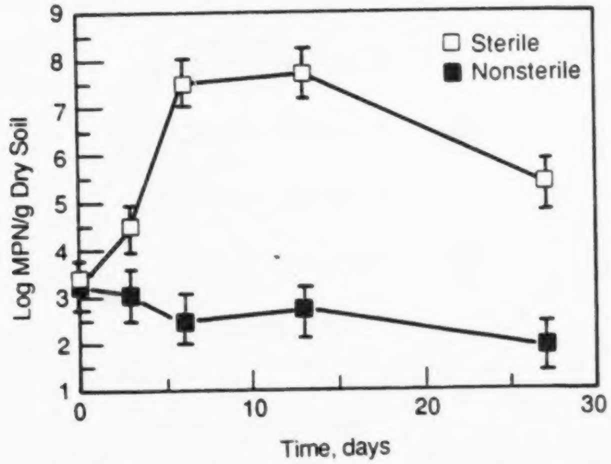

Figure 3. Populations of *P. putida* Tn*5* VI mutants in sterile and nonsterile Burbank soil as enumerated by MPN-DNA hybridization.

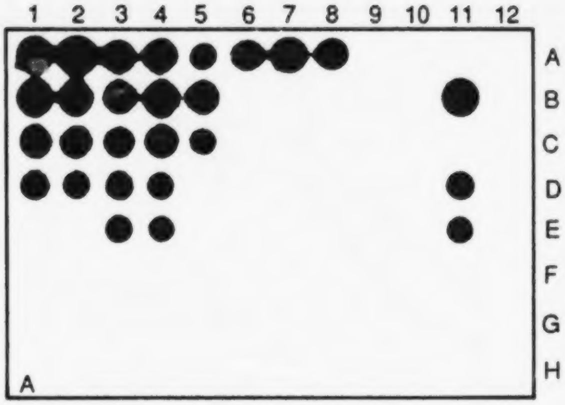

Figure 4. Autoradiograph of MPN-DNA filter probed with ^{32}P-labeled pGS9DNA. Columns 1 through 5 contain dilutions from sterile soil inoculated with *P. putida* Tn*5* and incubated for 27 days, and columns 6 through 10 are dilutions from nonsterile soil inoculated with *P. putida* mutants and incubated for the same period. Rows A through G are dilutions of $10^{-0.7}$ to $10^{7.7}$. Controls consist of sterile KB broth (A11), *E. coli* MV12 pGS9 (B11), *E. coli* C-1a (C11), *E. coli* WA803 pGS9 (D11), *P. putida* Tn*5* (E11), and *P. putida* wild type (F11).

Recovery of Tn*5* Bacteria from Soil

The sensitivity and limits of detection for Tn*5* VI mutants of *P. putida* and *Rhizobium* sp. by MPN-DNA hybridization was determined in two soils varying in texture and organic matter content. *P. putida* was positively identified in both Burbank and Palouse soils when added at 10 cells/g by this procedure (Figure 5). The number of cells added was within the 95% confidence limit of cells recovered by MPN spot blot. However, recovery values were consistently higher from the Burbank than from the Palouse soil, except at the highest concentration of cells added, in which case there was no difference.

Two species of rhizobia, *R. leguminosarum* biovar *viceae* (IP27A) and *R. leguminosarum* biovar *phaseoli* (Kim-5) containing Tn*5* and suicide plasmid sequences, were also evaluated for their recoverability from soil by MPN spot blot. As with *P. putida*, enumeration of rhizobia were in close agreement with the number of actual cells added except for *R. leguminosarum* IP27A where the recovery was 1.5 log units less than the 10^4 cells/g of soil added (Figure 6). The detection limit for the rhizobia in Burbank soil, even with antibiotic selection [kanamycin (Km) and kanamycin-rifampin (Km - Rm)], was 1 to 2 log units higher than for *P. putida* (Figure 5).

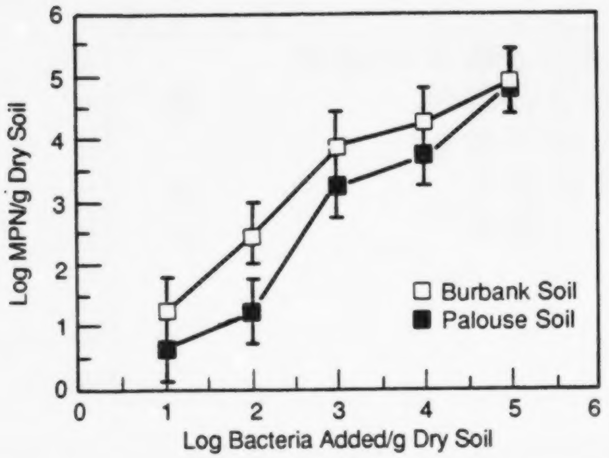

Figure 5. Recovery of *P. putida* cells containing Tn*5* added to soil by MPN-DNA hybridization. Populations of total bacteria were 6.88 and 8.30 log CFU/g of soil in Burbank and Palouse soils, respectively. Error bars represent 95% confidence limits.

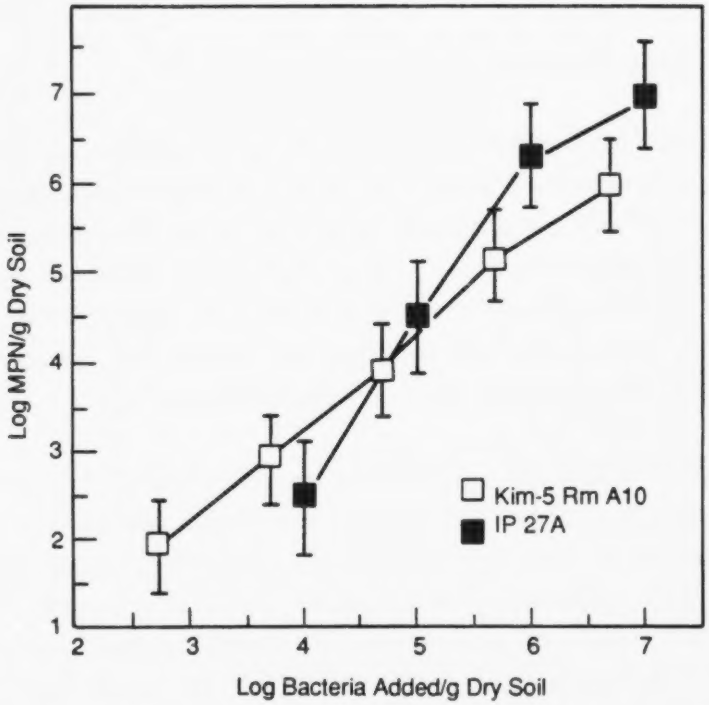

Figure 6. Recovery of *Rhizobium* spp. cells containing Tn*5* added to nonsterile Burbank soil by MPN-DNA hybridization.

Comparison of Enumerative Methods

Populations of *R. leguminosarum* biovar *phaseoli* (Kim-5) Tn*5* VI in autoclaved and nonsterile Burbank soil were compared using plate counts, MPN plant infectivity, colony blot DNA hybridization, MPN-DNA hybridization, and FA direct counts (Table 1). Recoveries of *R. leguminosarum* biovar *phaseoli* at zero time in sterile soil by plate count and in nonsterile soil by MPN replica plate were similar to the number of cells added. After 5 days of incubation, population values for all enumerative methods in sterile soil were statistically similar. However, by day 30, plate counts and colony blots estimated the population at 10^6 CFU/g dry soil, whereas FA counts and the MPN replica plate method estimated the population to be 10 fold higher, and approximately 10 fold lower, respectively.

Table 1. Enumeration of *R. leguminosarum* bv. *phaseoli* (Kim-5) Tn*5* VI from Sterile and Nonsterile Burbank Soil at 0, 5, and 30 days by Various Methods[a]

Method	Log Cells/g of Soil on Day: 5	Log Cells/g of Soil on Day: 30
Plate count	6.76 (0.04)	5.96 (0.66)
MPN plant infectivity	7.34 (7.97, 6.78)	NA
DNA hybridization		
Colony blot	6.81	6.00 (0.30)
MPN	6.51	NA
MPN replica plate[b]	7.00 (7.53, 6.51)	4.73 (5.23, 4.20)
Fluorescent antibody count	7.18 (0.23)	7.08 (0.48)

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[a] Actual cells added at zero time was 323 ± 43 per gram. Single values in parentheses are standard deviations; double values are the 95% confidence limits. NA, not assayed.

[b] Identical to MPN-DNA hybridization but with one transfer from microdilution plate to selective agar medium, followed by colony blot analysis.

Rhizobium leguminosarum biovar *phaseoli* (Kim-5) Tn*5* VI inoculated into nonsterile Burbank soil could not be detected by day 5. *P. putida* Tn*5* VI inoculated into nonsterile soil showed a population decline over time (see Figure 3), suggesting that *R. leguminosarum* also declined in numbers with time.

Enumeration by Associated Phenotypic Marker

Insertion of the transposon Tn*5* into the bacterial genome confers to the host kanamycin resistance, which may be used as an alternative to spontaneous antibiotic resistance. The efficiency of recovery from nonsterile soil of *R. leguminosarum* biovar *viceae* IP27A and *P. putida* Tn*5* VI mutants was assessed to determine the utility of transposon-associated drug resistance as a marker for the isolation and enumeration of gram-negative bacteria in soil.

Recovery of *R. leguminosarum* from nonsterile Palouse and Burbank soils using Km^r ranged from 44% to 85%, which was generally higher than for *P. putida*, which ranged from 10% to 33% (Table 2). Recoveries of *P. putida* were higher from Burbank than from Palouse soil, while no trend for differences in recoveries because of soil type was evident for the *R. leguminosarum* biovar *viceae* isolate. Colony blots probed with ^{32}P-labeled pGS9 DNA indicated that 100% of the colonies that grew on either YEM agar containing 100 μg/ml each of kanamycin sulfate, cyclohexamide, and pimaricin, or on the medium of Sands and Rovira (30) amended with 100 μg/ml of kanamycin, at the dilutions counted, contained Tn*5*.

DISCUSSION

The use of gene probes for the detection of specific bacterial genotypes and virus particles in environmental samples has been shown to be widely applicable (3,19,31). Enumeration by MPN-DNA hybridization is based on dilution-to-extinction probability theory (1) and does not rely on counting individual colonies or spots in the case of plate counts or colony blot hybridization. The MPN procedure proved to be very sensitive and accurate for the enumeration of Tn*5* VI bacteria in soil; approximately 10 cells/g were readily enumerated for *P. putida*. This level of detection is comparable with that obtained by other sensitive methods for following recombinant bacteria in the environment, including expression of cloned *lac* genes (10) and spontaneous antibiotic resistance (37). In this paper, a five-replicate (microdilution well) MPN was used, but the sensitivity and accuracy could be further improved by increasing the number of replicates (29).

Table 2. Recovery of Tn*5* VI Isolates from Nonsterile Soil by Transposon-Associated Phenotypic Marker (Km^r)

Isolate Soil Type	Log CFU/g of Soil (SD)		
	Cells Added	Cells Recovered	% Recovery
R. leguminosarum	6.23	6.09 (5.27) (a)	72
bv. *viceae*			
IP27A	4.23	4.15 (3.56)	83
Burbank	2.71	2.51 (1.81)	63
R. leguminosarum	6.23	6.16 (5.40)	85
bv. *viceae*			
IP27A	4.23	3.88 (3.20)	44
Palouse	2.71	2.54 (2.25)	68
P. putida	5.78	5.30 (4.76)	33
Burbank	3.78	3.30 (2.70)	33
	2.26	1.78 (1.08)	33
P. putida	5.78	5.05 (4.80)	19
Palouse	3.78	3.27 (2.40)	31
	2.26	1.27 (1.05)	10

The utility of DNA probes for the detection and enumeration of specific microorganisms and genotypes in soil is dependent on the availability of a unique, specific probe. The problem of specificity is enhanced when the target organism is present in soil at low numbers, which increases the background homology with indigenous soil bacteria. These studies involve the use of a whole plasmid (pGS9) probe to detect Tn*5* and plasmid vector sequences located in the bacterial chromosome. Our findings indicate that the sensitivity of detection by MPN-DNA hybridization is significantly increased when the target includes pCU101 vector (33) sequences (24.5 kilobases) in addition to Tn*5* sequences (5.5 kilobases). However, the use of VI mutants for microbial ecology studies may be undesirable. Because pCU101 is a recombinant plasmid, federal agency approval would be necessary before field testing of VI mutants. In addition, vector sequences appear less stable than Tn*5* under soil conditions and could reduce competitiveness in an organism because of an increase in genetic load. Studies are currently being conducted in our laboratory to assess the fate of bacteria containing only Tn*5* in soil-core microcosms.

The MPN procedure should prove useful for enumerating recombinant bacteria in field-test situations, but care must be taken in the selection of a specific probe as well as obtaining control samples before the release of the organisms for determination of possible background homology with indigenous soil bacteria. In our studies, nonspecific binding of probe to soil colloids or hybridization with DNA of indigenous soil bacteria was not a problem. However, nonsterile soil consisted of air-dried soil moistened before use. Although moistening of air-dried soil typically results in a flush of microbial growth and activity, the composition of the microflora may be radically different from fresh soil. Further studies are required to determine whether the whole pGS9 probe is specific enough for detection of Tn*5* bacteria in fresh soil.

In addition to detection and enumeration by DNA hybridization, phenotypic expression of the Tn*5* Km^r gene allows for enumeration of Tn*5* mutant bacteria in soil as described in this study and elsewhere (36). Although Tn*5* has been shown to confer resistance to streptomycin in addition to kanamycin in some gram-negative bacteria (27), we did not use this as an additional marker in our studies because the levels of resistance conferred were not high enough to aid in selecting against indigenous soil microflora. However, the utility of transposons as either phenotypic (antibiotic resistance) or genotypic (DNA probes) markers for experiments in microbial ecology will depend on the location and relative stability of the transposon within the bacterial genome. The results presented indicate that Tn*5* can transpose into the chromosome of both *R. leguminosarum* and *P. putida*, and that the Km^r marker is stable after growth for 33 generations in sterile soil. However, donor plasmid sequences (pGS9), which integrated in addition to Tn*5*, were less stable in *R. leguminosarum* yet had about the same degree of stability as Tn*5* alone in *P. putida*. The 5% frequency with which donor plasmid sequences were observed in Km^r mutants in this study is similar to 3% observed for Tn*5* mutagenesis with rhizobia (33) and 3.5% observed for *Erwinia chrysanthemi* (7).

Precise excision of Tn*5* from rhizobia has been shown to occur at a frequency of 10^{-8} to 10^{-9} in pure culture (26,33) and is therefore a relatively rare event. However, in these studies with *P. putida* the loss of Km^r in sterile soil was as high as 10^{-2} and may reflect the instability of VI mutants under soil conditions. In pure culture this frequency may vary because Km^r, conferred by Tn*5*, in *P. fluorescens* has been shown to be stable for more than 50 generations (36). Tn*5* is also capable of transposing to a number of different target sites in the genome of *P. putida (5)*, and the stability of Tn*5* insertion in these different regions may vary. The use of

Tn*5* or other transposons as markers for bacteria will require biochemical and physical genetic analysis on an individual basis to ensure that a stable, ecologically competent mutant is selected.

A number of factors will influence the sensitivity of the MPN-DNA hybridization procedure. As with a variety of detection and enumeration methods, including plate counts and colony blot hybridization, culturability of the organism being enumerated is necessary. While a selective medium is desirable and can increase sensitivity, it is not a necessity for all organisms. *P. putida*, enumerated by MPN-DNA hybridization, was cultured in KB broth in the absence of antibiotic selection. For bacteria such as *Rhizobium* spp., which grow more slowly (generation time ≈ 1,000 min) relative to most soil *Pseudomonas* spp. (generation time ≈ 45 min), a more selective medium is required. Even with antibiotic selection the detection limit for *Rhizobium* spp. cells in soil was approximately 10^2/g. The dependence on culturability can be a problem, as it has been shown that *Vibrio cholerae* and some related pathogens can survive in a viable but nonculturable state in a marine environment (8). Although FA counting can be used to detect and enumerate such bacteria in soil, it may also be enumerating cells which are no longer viable, yet whose outer membrane antigenic determinants remain intact, especially in dry soil. Time-course enumeration of Kim-5 Tn*5* VI in soil in this study (see Table 1) shows that population estimates by FA counting are 10- to 100 fold higher after 30 days in soil than by methods that require culturing. Previous work by Bezdicek and Donaldson (4) also showed higher estimates of bacterial populations by FA counting than by culturing and MPN.

Interactions between microbial cells and clay and organic matter may also account for observed differences in cell recoveries between the two soils. Relative recovery by MPN-DNA hybridization of identical numbers of *P. putida* Tn*5* VI cells added to Burbank and Palouse soils was consistently higher from the Burbank than from the Palouse soil (see Figure 5). The Palouse soil has a significantly higher percentage of both clay and organic matter than the Burbank soil, which suggests that binding of cells to soil colloidal particles is limiting recovery. These observations are in agreement with Stotzky and Burns (34), who cited empirical observations such as lack of leaching of microbes through the soil profile during periods of heavy rain, failure to wash substantial numbers of cells from soil in perfusion or leaching experiments, and increased release of microbes from soil upon sonication or treatment with surfactants as strong evidence for the binding of microorganisms to soil particles. Bacterial interactions with soil colloidal particles may also account for the relative differences in recovery on selective medium (Km) between *R. leguminosarum* and *P. putida* (see Table 2) and for the lack of recovery values that approach 100% for either soil. Bacterial isolates vary both in their electrokinetic potential because of chemical differences in cell wall components and in their ability to produce substances that increase their adherence to surfaces (24).

The differences observed for growth and survival of both *P. putida* (see Figure 3) and *R. leguminosarum* biovar *phaseoli* Kim-5 Tn*5* VI (see Table 2) in sterile and nonsterile soil emphasize the need for studies on the growth and survival of recombinant microorganisms to be conducted under environmental conditions that simulate (as closely as possible) the actual environment into which the microorganisms will be released. Microbial growth and survival in an ecosystem are dependent on abiotic (e.g., pH, Eh, water potential, nutrient availability) and biotic factors (e.g., predation, bacteriostasis). Although microbial survival studies in sterile soil are conducive to recovery and enumeration of the test organism, differences in survival patterns (22) do not allow extrapolation of results to predict survival in the environment.

The use of DNA probes was the focal point of this work; however, a variety of standard cultural methods as well as new developments in detection technology will be required for sensitive environmental detection and enumeration of bacteria. A major advantage in using gene probes in conjunction with other methods is that they are specific for a gene regardless of host and can be used for detection even if no selective phenotypic marker is available or expressed.

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6.0 CONCLUSIONS

Microcosm research has a long history that has resulted in nearly as many types and designs of microcosms as there are research papers involving microcosms. However, they all share (or should share) at least one common element; that is, they represent, at some defined ecosystem level, the interactions of organisms with each other and their environment. Most work conducted with microcosms to date has been directed to evaluate the fate and impact of chemicals in the environment, including effects on individual species and ecological processes. Although there may be some similarities between chemical and GEM fate and effect studies (i.e., transport mechanisms, ecologic effects), there are many fundamental differences. Among these fundamental differences are the ability of microorganisms to replicate and increase in population size as well as exchange genetic information.

The research presented in this document, which represents the first scientific results on the fate and transport of GEMs in terrestrial microcosms, does have limitations. Therefore, some of the techniques and results discussed will not be directly applicable to biotechnology risk assessment without further work. However, the research findings presented herein, in combination with recognized limitations of microcosms, provide insight into the issues of biotechnology risk assessment to which microcosms can be immedaitely applied - fate, survival, and transport. They also provide directions for future research regarding those aspects of risk assessment that require futher development, such as ecological effects. Some of these limitations, which have plagued microcosm chemical fate research for several decades, include the effects of sizing and scaling, disturbances caused by sampling, and lack of calibration with the field.

The advantages and limitations to the research presented in this document have been clearly recognized and presented by the individual authors and provide a focal point for a critical evaluation of the individual sections. In Section 2.0, Assessing the Persistence of Recombinant Bacteria in Microcosms, it was demonstrated that simple (potting soil composed of peat mix and perlite, lacking environmental control and monitoring) and complex microcosms (soil with partial control and monitoring of environmental conditions) were useful as tools for preliminary assessments of microbial viability in terrestrial agricultural ecosystems. This was supported by the results showing the survival patterns of *Enterobacter cloacae* (pBR322) in soil and on plant surfaces and the ingestion of this same microorganism by cutworms and survival in the foregut and frass. It was emphasized that these results were preliminary and that microcosm results need to be calibrated with field studies. The studies presented were relatively short term, and the desirability of long-term evaluations (3 months and longer) was emphasized. An initial attempt was made in this work to evaluate the effect of moisture (i.e., soil moisture and relative humidity) on microbial fate in microcosms. It was suggested that to obtain a more mechanistic understanding of how environmental variables such as moisture, temperature, radiation, etc. impact microbial survival in terrestrial environments, further work is needed in microcosm systems in which environmental variables can be more stringently controlled. Finally, to determine the effect that genetic engineering may have on a microorganism's survival and competitive ability, comparisons between GEMs and nonrecombinant parent strains are needed.

In Section 3.0, The Fate of Recombinant Pseudomonads in Modified Soil-Core Microcosms (Versacores), the Versacore microcosm design was used to monitor the fate and competitiveness of genetically engineered bacteria in soil. Both selective media and gene probes were used to follow the population dynamics of two recombinant bacteria introduced into Versacore microcosms. The major advantage to this design was the relative ease of spatial and temporal sampling, allowing an evaluation of the movement of bacteria by water percolation and with plant roots. The major limitations to using repacked soil columns as microcosms are 1) mixing and repacking soil changes the microbial environment by releasing nutrients which were physically unavailable prior to mixing; and 2) the natural structure and pore channels of the soil are disrupted. These effects could influence both microbial survival and transport through the soil. It was suggested that in experiments in which natural transport is being measured, that intact soil cores may be more appropriate. A major conclusion of this research was that the Versacore microcosms were appropriate for tracking recombinant bacteria and determining the effect of different environmental parameters on survival and dissemination. It should be emphasized that mixing and repacking soil mimics the agronomic practice of tillage.

In Sections 4.1, Intact Soil-Core Microcosms for Evaluating the Fate and Ecological Impacts from the Release of Genetically Engineered Microorganisms, and 4.2, Fate of Root Growth-Inhibiting *Pseudomonas* sp. Tn5 Mutants in Intact Soil-Core Microcosms, the authors utilized intact soil cores (17 cm diameter by 60 cm depth) as terrestrial microcosms for evaluating the fate and transport of genetically altered bacteria. The major limitations to the research presented in Section 4.1 are 1) the limited movement in and colonization of the soil cores by *Azospirillum lipoferum* transposon mutants, and 2) the inability to detect the introduced microorganism at populations less than 10^3/g soil. These limitations made a critical evaluation of the appropriateness of this microcosm design for Biotechnology Risk Assessment difficult. However, this research did indicate that intact soil cores retained a high degree of ecosystem complexity and therefore may more closely mimic undisturbed field plots. In addition, a number of methods were developed to allow evaluation of vertical movement of GEMs with water and plant roots, GEM ingestion by insects, movement in plant xylem, and nutrient export as leachate.

In Section 4.2, tox^+ and tox^- Tn*5* mutant root growth-inhibiting *Pseudomonas* sp. were evaluated for fate, transport, and effects in intact soil-core microcosms. The level of detection was much improved for these microorganisms (approximately 20 CFU/g soil) and their survival and colonization in various compartments of the microcosm (i.e., bulk soil, rhizosphere with depth, and earthworms) were more extensive than was observed in earlier experiments *Azospirillum lipoferum* strains. The findings of this research indicate that intact soil-core microcosms may be useful tools for prerelease evaluation of GEMs. An advantage to this microcosm design is that they are site specific; that is, soil cores can be taken from the location where the release is planned and used to obtain prerelease information on fate and effects. The authors suggested that the major limitation of this study was a lack of calibration with the field. Without some type of field calibration there is no way of knowing to what degree the information being gained from microcosms represents what would or could occur in the field. Although at this time microcosm data can provide information on the fate, survival, and potential effects from a particular GEM, it would be inappropriate to base a release decision solely on microcosm data.

Section 5.1, Detection of Two Engineered Pseudomonads and a Spontaneous Deletion Recombinant Derivative in Soil Using Gene Probes, and Section 5.2, Enumeration of Tn*5* Mutant Bacteria in Soil by MPN-DNA Hybridization and Antibiotic Resistance, present two recent developments in the application of DNA probes to detection and enumeration of bacteria in terrestrial environments. In Section 5.1, a direct DNA extraction method, slightly modified from that previously described by Holben et al. (2), was used to detect and distinguish between two genetically engineered pseudomonads in the same soil. The utility and sensitivity of this method was demonstrated by the detection of a deletion recombination event in an engineered *Pseudomonas cepacia* strain released to the soil. The major limitations to this method are the poor sensitivity (approximately 10^4 cells/g soil) and the intensive labor required relative to standard cultural methods. The latter could limit the application of this method to microcosm and field studies that involve numerous spatial and temporal samplings. The sensitivity of the direct DNA hybridization analysis method has already been improved by Steffan et al. (3), who used *Taq* polymerase chain reaction to amplify specific bacterial sequences in a heterogeneous mixture of DNA and achieved a detection limit of approximately 1 cell/g soil. This modification of the DNA probe technique (2) can be applied to microcosm analyses for greatly improved detection capabilitites.

In Section 5.2, a most-probable-number DNA hybridization procedure used to enumerate GEMs in environmental samples is described. The major advantages of this technique included the ability to quantify GEMs and the ability to detect as few as 10 cells/g soil of a *Pseudomonas putida* Tn*5* mutant. Enumeration results using this method agreed well with plate counts on selective medium and direct counts using fluorescent antibodies. However, this method depends on being able to culture the target organism and therefore would not detect viable but nonculturable bacteria or free DNA that may be in soil.

The studies presented in this report demonstrated that terrestrial microcosms are appropriate tools for evaluating fate, survival, transport, and genetic rearrangement of GEMs released to the terrestrial environment. Some major conclusions can be drawn from this report:

1. Terrestrial microcosms are an appropriate tool for biotechnology risk assessment and microbial ecology research, provided the operator understands the limitations associated with a particular microcosm design.

2. Major advantages to the use of microcosms for biotechnology risk assessment are the ability to control and monitor environmental conditions and to contain the organisms in a model environment..

3. DNA probes can be highly specific and sensitive tools for monitoring the fate of GEMs and their recombinant DNA in terrestrial environments, but are currently limited principally by the intensive labor and time required by standard culturing techniques.

4. Field studies are necessary to calibrate results obtained using microcosms with the field to fully demonstrate the utility of microcosms for predicting GEM behavior in the field.

The approaches to terrestrial microcosm technology discussed here have ranged from the use of excised intact soil cores to the use of soil in commercial trays or pots to a microscale setup using clear plastic tubing. In general, the larger scaled microcosms are more adaptable and appropriate for studying fate and transport phenomena than are the small microcosms. However, all constructs provided unique information regarding the ecological competitiveness, survival, and fate of recombinant bacteria and therefore can be used for these aspects of risk assessment. Microcosms were also shown to have utility for developing and testing new methods, such as the use of DNA probes for detecting and enumerating GEMs in complex media (i.e., natural soils). It should be emphasized, however, that it is critical that the investigator or operator recognize the operational and ecological limitations of the particular microcosm being used, and not attempt to answer questions or make extrapolations that transcend the recognized limitations of the test system.

This synthesis document is targeted for use by the U.S. Environmental Protection Agency's Office of Toxic Substances and Office of Pesticide Programs for identifying microcosm biotechnology risk assessment applications (i.e., fate, survival, and transport) and for the Agency's Office of Research and Development for identifying future microcosm research needs. It is also intended for general use by industry and the scientific community wherever information is needed on the use of terrestrial microcosms relevant to the assessment of recombinant microorganisms.

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